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13. ABSTRACT ***(Maximum 200 Words)***

Epidemiologic studies have repeatedly demonstrated that women who undergo a first full-term pregnancy early in life have a significantly reduced lifetime risk of breast cancer. Similar to humans, rodents also display parity-induced protection against breast cancer. We have used high-density DNA oligonucleotide microarrays to identify genes whose expression in the murine mammary gland is persistently altered as a consequence of an early first full-term pregnancy. The panel of differentially expressed genes that we have isolated reproducibly distinguishes between the nulliparous and parous states of the mammary gland in multiple strains of mice and rats. We find that parity results in the persistent down-regulation of multiple genes encoding epithelial growth factors as well as the persistent upregulation of growth-inhibitory pathways. Our studies further indicate that parity results in a persistent increase in the differentiated state of the mammary gland as well as persistent changes in the immunologic environment and hematopoietic cell types resident within the gland. These data provide the first molecular description of a developmental state of the mammary gland that is associated with reduced cancer risk and suggest novel hypotheses for the mechanistic basis by which parity may modulate breast cancer risk.

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Submitted manuscript:

Celina M. D'Cruz *[1], Susan E. Moody *[1], Stephen R. Master[1], Jennifer Hartman[1], Elizabeth A. Keiper[1], Marcin B. Imielinski[1], James D. Cox[1], James Y. Wang[1], Seung I. Ha[1], Blaine A. Keister[1] and Lewis A. Chodosh[1,2,3] Early First Full-Term Pregnancy Results in Permanent Changes in Gene Expression in the Murine Mammary Gland.

INTRODUCTION

Epidemiologic studies have highlighted the relationship between hormones and carcinogenesis in the breast by identifying endocrine risk factors for breast cancer that are related to the timing of reproductive endocrine events, such as menarche and menopause. This relationship is illustrated by the observation that women who undergo an early first full-term pregnancy have a significantly reduced lifetime risk of breast cancer. The recognition that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that events associated with a decrease in breast cancer risk, such as early first full-term pregnancy, might be mimicked pharmacologically. As such, understanding the mechanisms by which these events influence breast cancer risk would facilitate the design of safe and effective hormonal chemoprevention regimens. In addition, the testing of such regimens would be facilitated by the identification of biomarkers that accurately reflect early biological changes in the breast associated with reproductive endocrine events that alter breast cancer susceptibility. Since the impact of reproductive endocrine history both on mammary gland development and on breast cancer risk most likely results from differential effects of hormones on particular subpopulations of cells, understanding these changes will almost certainly require a thorough understanding of the cell types present in the breast and the manner in which hormones affect their normal programs of differentiation and development. Unfortunately, pursuit of this goal has been hampered by the fact that few, if any, molecular biomarkers have been identified to date that are specific for changes in the breast that occur as a result of reproductive endocrine events known to influence breast cancer risk. Such biomarkers are essential both for understanding the impact of reproductive history on mammary epithelial biology in general, and the molecular and cellular basis of parity-induced protection against breast cancer in particular, as well as for the rational design and testing of hormonal chemoprevention regimens aimed at mimicking this naturally occurring protective event.

We hypothesize that an early first full-term pregnancy results in a permanent change in the breast that confers a decreased risk for the subsequent development of breast cancer. We further hypothesize that parity-induced changes in the number and distribution of specific epithelial cell subtypes in the breast may contribute to parity-induced changes in breast cancer risk. To address these hypotheses, the specific aims of this proposal were designed to identify and evaluate molecular biomarkers for parity-induced changes in the breast by isolating genes that are differentially expressed between the parous and nulliparous rodent breast using DNA chip technology. Each of the technical objectives and tasks proposed have been completed on schedule.

In this report we have used high-density oligonucleotide microarrays to analyze the impact of early first full-term pregnancy on global gene expression profiles within the murine mammary gland. This approach has led to the identification of a panel of genes whose expression in the mammary gland is persistently altered as a consequence of parity in multiple strains of mice as well as in two widely used rat models for parity-induced protection against breast cancer. Our findings demonstrate that parity induces the persistent downregulation of multiple genes encoding epithelial growth factors as well as the persistent upregulation of the growth-inhibitory molecule, *TGF-β3*, and several of its downstream targets. Our findings further indicate that parity results in a persistent increase in the differentiated state of the mammary gland as well as permanent changes in the hematopoietic cell types resident within the gland. These findings define a developmental state of the mammary gland that is refractory to carcinogenesis and suggest novel hypotheses for mechanisms by which parity may modulate breast cancer risk. We believe that these studies represent an important step towards determining the mechanisms by which breast cancer susceptibility is modulated by reproductive history.

BODY

At the outset of this study, we hypothesized that generating a sufficiently robust panel of parous- and nulliparous-specific markers would require a powerful parallel screening technique that would permit the sensitive, reliable and reproducible detection of differentially expressed genes. To accomplish this aim, we chose to determine gene expression levels by hybridization of mRNA to high-density oligonucleotide arrays. This approach has turned out to be extremely successful. While several of the highlights of our experimental studies are presented in this report, the complete results obtained are described in the accompanying manuscript from my laboratory entitled,"Early first full-term pregnancy results in permanent changes in gene expression in the murine mammary gland" by D'Cruz et al. This manuscript has been submitted for publication. Please refer to this manuscript for all details.

Technical Objective I: Isolate genes expressed in the breast in a parity-specific manner.

To determine whether our model system behaved similarly to other established systems for parity-induced protection against breast cancer, we initially compared the morphology of mammary glands from rodents that have undergone a single-round of pregnancy, 21 days of lactation and 4 weeks of regression to the morphology of mammary glands from nulliparous littermates (Fig. 1). It has previously been noted that mammary glands of parous woman have more differentiated lobules than seen in nulliparous woman[1]. Whole mount analysis of mammary glands from virgin and parous

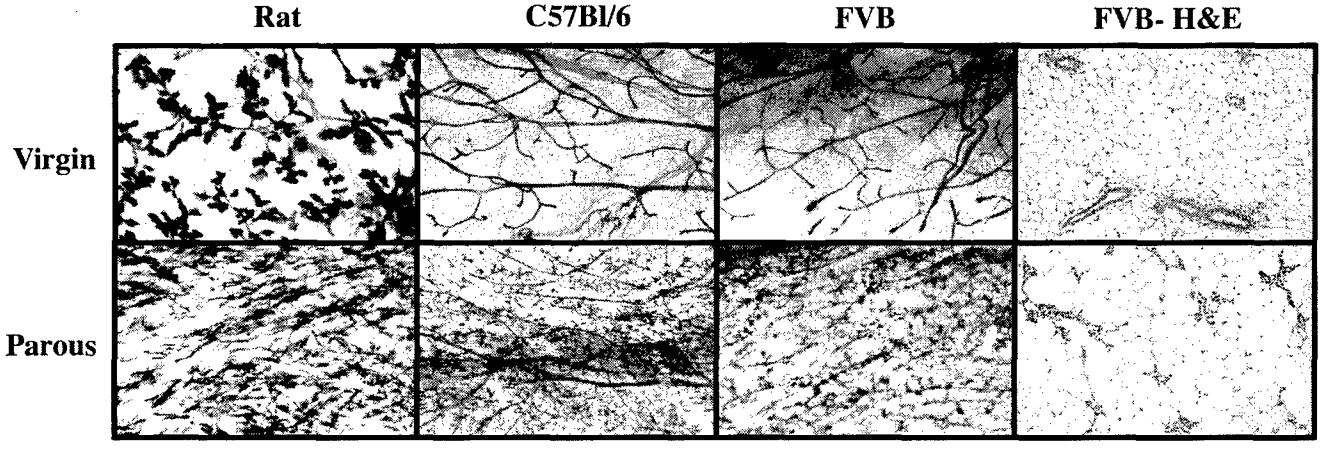

Figure 1: Parity-induced changes in mammary gland morphology

Sprague-Dawley rats demonstrate marked differences in the architecture of the virgin and parous glands. The parous gland is more highly branched, suggesting that it is permanently altered by parity-related changes. We have also examined two independent strains of mice, C57BL/6 and FVB to determine whether these morphological features appear in the murine system as well. Parous animals were mated at 4 wks of age, a time corresponding to puberty in order to approximate "early-parity". Although the virgin murine glands appear less developed as compared to the rat, similar overall differences are observed between the virgin and parous glands of mice and rats. Again the parous gland is more highly branched (Fig 1). Analysis of H&E stained glands demonstrate that there appears to be roughly the same amount of epithelium, an observation that has been confirmed by quantitation of epithelium by Cytokeratin 18 expression levels. The structural changes which accompany parity in the mammary gland appear to be permanent as the differences in the gland remain for essentially the life of the animal (data not shown).

Changes in the breast that are induced by reproductive events such as parity are likely to involve complex changes in the expression of multiple genes. Many techniques for comparing mRNA populations, such as differential display, subtractive hybridization, and serial analysis of gene expression (SAGE) have proven to be labor-intensive and relatively insensitive, particularly for genes expressed at very low levels. In addition, these techniques typically generate high percentages of false-positive clones. As such, we have chosen a technical approach that permits the direct assessment of mRNA expression profiles from large numbers of genes in parallel. This approach, referred to as Affymetrix GeneChip technology, involves the determination of the relative abundance of mRNAs based on hybridization of complex mRNA populations to high-density arrays, or "chips" containing arrayed synthetic oligonucleotides[2-4]. Each chip is composed of 65,000 different oligonucleotides of defined sequence which are synthesized directly on derivatized glass slides. Each gene is represented on the array by 20 specific, unique 25-mer oligonucleotide probe pairs, such that each chip permits the detection of approximately 1,600 genes. A combination of arrays permits expression levels to be determined for more than 6,800 full-length genes in the UniGene, GenBank and TIGR databases. mRNA isolated from a sample of interest is used to generate an antisense poly(A)$^+$ probe with incorporated biotin-labeled nucleotides. This mRNA probe is then hybridized to the array using the GeneChip fluidics station which automates sample introduction, hybridization, incubation, and washing. Quantitative detection of mRNA bound to each oligonucleotide cell is achieved by measuring fluorescence of bound phycoerythrin-streptavidin using a Hewlett-Packard GeneArray scanner. Finally, the GeneChip operating system software automatically calculates intensity values for each probe cell. Expression for each gene is determined by analyzing the extent of binding to the 20 pairs of oligonucleotides specific for that gene. Fluorescence intensity from the hybridized arrays directly correlate with mRNA abundance levels. The GeneChip instrumentation system necessary for these experiments has recently been purchased from Affymetrix, Inc. by the Institute for Human Gene Therapy. This equipment will be located within the Chodosh laboratory.

GeneChip technology permits the quantitative, sensitive, specific and reproducible determination of gene expression profiles [2-4]. A high percentage of clones identified are true positives, thereby minimizing subsequent labor invested in pursuing false leads. Differences in expression levels of approximately 2 to 2.5-fold can be reliably distinguished. The detection limit is approximately 2 - 5 copies per cell, and is quantitative over three orders of magnitude [2-4]. Each array contains probes comlementary to multiple reference genes that permit data within and among experiments to be normalized and quantitated [2-4]. In addition, multiple internal reference points permit the normalization and quantitation of data from different experiments. Repeat hybridization with the same probe is reported to yield only one in 3,000 genes that differ by more than 3-fold [2-4]. Hybridization with probes independently prepared by different operators from the same sample source is reported to yield less than 1 in 1,000 genes that differ by more than a factor of three. Compared to array-based methods that involve the spotting of cDNAs onto nylon membranes, an additional advantage of the oligonucleotide-based approach is that highly specific binding and detection can be achieved even within conserved gene families. Finally, this parallel approach lends itself to increases in scale to permit large numbers of genes to be monitored and is thereby ideally suited for the extensive comparison of complex mRNA populations such as were expected in this proposal.

Task 1: *Months 1-12: Generation of mRNA samples representing reproductive endpoints.*

This task has been completed on schedule. Inbred FVB mice (which do not harbor active MMTV provirus) were bred and maintained in a barrier facility with a regular 12 hour light/12 hour dark cycle to facilitate regular estrous cycling. Animals were given food and water *ad libitum*. Mice were housed 4

per cage during virgin development and postlactational regression, and individually during pregnancy and lactation. Nulliparous controls were housed identically to parous animals. Daily vaginal smears were performed on animals to document regular cycling. Total RNA was isolated from the mammary glands of mice that had either undergone an early first full-term pregnancy followed by 3 weeks of lactation and 4 weeks of postlactational regression, or that were maintained as age-matched nulliparous controls. Animals were euthanized by carbon dioxide asphyxiation at specified developmental time points, and the number 4, 5 and 6 mammary gland pairs were harvested and snap frozen in liquid nitrogen, following removal of the lymph node embedded in gland 4. Mammary tissue from each animal was also fixed in 10% neutral buffered formalin and embedded in paraffin for *in situ* hybridization studies. Total RNA was isolated from tissues by homogenization in guanidinium isothiocyanate, followed by ultracentrifugation through cesium chloride. In total, RNA was harvested from parous and nulliparous animals in three independent experiments with each experiment comprised of 20 parous and 20 nulliparous animals. Additional breeding of animals is ongoing in order to generate developmental time points representing other reproductive variables such as early vs. late first full-term pregnancy and duration of postlactational regression.

Task 2: *Months 6-18: GeneChip-based screening.*

This task has been completed on schedule. Standard Affymetrix protocols were used to generate and hybridize probes prepared from nulliparous and parous RNA samples, and to quantitate and analyze gene expression levels. RNA isolated from nulliparous and parous animals was subject to reverse transcription to make first strand cDNA that incorporated a T7 RNA polymerase promoter at the 3' end. Following completion of the second strand of cDNA, this material was used in an in vitro transcription reaction to generate biotin-labeled cRNA representing each experimental pool of mammary gland RNA. This in vitro transcribed cRNA was then fragmented to facilitate hybridization. Hybridization reactions were prepared for each sample using fragmented cRNA in addition to standardized internal controls. Hybridization reactions representing each of the 3 nulliparous samples as well as each of the 3 parous samples were hybridized to murine 6500 A, B, C and D oligonuleotide arrays, representing approximately 5500 murine genes.

Using GeneChip software, expression levels were calculated for each of the 5500 genes represented on the Mu6500 chips for all six samples. This information was imported into a software database. Confidence intervals were generated for each gene in order to identify genes that were differentially expressed between the nulliparous and parous states at a confidence level > 90%. In addition, Affymetrix algorithms were applied to each individual nulliparous/parous pair to identify those genes that whose expression was considered to increase or decrease. This analysis was repeated for each of the three experiments. Genes that were considered to either increase in all three experiments or decrease in all three experiments were identified. This approach, combined with Northern analysis of candidate genes identified by microarray analysis as being differentially expressed in two out of three sets of nulliparous and parous samples, resulted in the identification of 41 differentially expressed genes (Table 1). These include 10 genes that are preferentially expressed in the nulliparous mammary gland as well as 31 genes that are preferentially expressed in the parous mammary gland. Notably, expression levels of the epithelial cell marker, *cytokeratin18*, do not differ between the nulliparous and parous mammary gland, indicating that the observed changes in gene expression are not merely a consequence of the expansion or contraction of the epithelial cell compartment (Fig. 2).

Table 1: Differentially Expressed Genes

Genes Down-Regulated

Sequence Identity	Accession Number	Function	Avg. Fold Change	Northern
Amphiregulin	L41352	Growth Factor	2.8	Y
Pleiotrophin	D90225	Growth Factor	2.0	Y
Insulin-Like Growth Factor 1B	W10072	Growth Factor	2.2	ND
Insulin-Like Growth Factor 1A	X04480	Growth Factor	1.6	Y
Thyroid Stimulating Hormone Receptor	U02602	Hormone Receptor	2.0	Y
Leptin	U18812	Hormone	2.0	Y
Ig Superfamily Containing Leucine-rich repeat	AA059664	Cell Adhesion	2.5	Y
MUC 18	AA088962	Cell Adhesion	2.0	Y
Superoxide Dismutase III (SOD3)	X84940	Oxidoreductase	2.5	Y
Carbonic Anhydrase III	M27796	Hydratase	1.4	Y

Genes Up-Regulated

Sequence Identity	Accession Number	Function	Avg. Fold Change	Northern
Whey Acidic Protein	J00544	Differentiation/Milk Protein	24	Y
Gamma-Casein	D10215	Differentiation/Milk Protein	20	Y
Alpha-Lactalbumin	M80909	Differentiation/Milk Protein	13	Y
Lactoferrin	J03298	Differentiation/Iron Transport	4.8	Y
Alpha-Casein	M36780	Differentiation/Milk Protein	4.2	ND
WDNM1	X93037	Differentiation/Protease Inhibitor	3.5	Y
Beta-Casein	X04490	Differentiation/Milk Protein	3.3	Y
Kappa-Casein	M10114	Differentiation/Milk Protein	2.9	Y
Adipocyte Differentiation Related Protein	M93275	Differentiation	1.8	Y
Carboxyl Ester Lipase	U37386	Lipid Degradation	6.6	Y
LPS-Binding Protein	X99347	Antibacterial/Milk	6.5	Y
Lysozyme P	M21050	Antibacterial/Milk	2.0	ND
Immunoglobulin M Heavy Chain	ET61785	Immunoglobulin	30	Y
Immunoglobulin G Heavy Chain	ET61798	Immunoglobulin	13	Y
Immunoglobulin A Heavy Chain	J00475	Immunoglobulin	21	Y
Kappa Light Chain	X16678	Immunoglobulin	7.0	Y
Macrophage Metalloelastase	M82831	Metalloprotease	2.1	Y
Macrophage Expressed gene 1 (Mpeg1)	L20315	Cell Signalling	1.7	Y
Lipocalin 2	W13166	Cell Signalling	2.6	Y
T-cell death associated gene (Tdag)	U44088	Cell Signalling	2.3	Y
Transforming Growth Factor Beta-3	M32745	Growth Inhibition	1.9	Y
Clusterin	L08325	Apoptosis/TGF-ß Pathway	2.0	Y
Eta-1	X16151	Cell Signalling/TGF-ß Pathway	8.8	Y
Id-2	M69293	Cell Cycle/TGF-ß Pathway	2.3	Y
Carbonic Anhydrase II	K00811	Hydratase	3.0	Y
Cyclin D1	A1849928	Cell Cycle	1.8	Y
CRBP1	X60367	Retinol-Binding	3.5	Y
Connexin 26	M81445	Gap Junction	3.1	Y
Folate-Binding Protein 1	ET63126	Receptor	2.6	Y
Adenosine Deaminase	M10319	Nucleotide Metabolism	3.0	Y
Chitinase 3-like 1 (BRP-39)	X93035	Glycoprotein	3.2	Y

Table 1: Genes found to exhibit a persistent change in expression in fully involuted compared to age-matched nulliparous mammary glands. Genes whose differential expression was confirmed by Northern analysis are indicated (Y). Genes whose differential expression was not tested (ND) by Northern hybridization were listed if they displayed a consistent change in regulation in 3/3 microarray experiments.

Task 3: *Months 6-20: Testing of candidate genes by Northern hybridization and RNase protection.*

This task has been completed on schedule. In order to test the differential expression of the candidate genes that we had identified, we generated 400-600 base pair fragments from the designated genes using RT-PCR and probed Northern blots containing one virgin and one parous sample identical to material that was used in the GeneChip analysis, as well as two independent virgin and parous

samples to control for biological variability. This approach confirmed the differential expression of all of the genes in which expression either increased or decreased in all three parous/nulliparous sets, as well as many of the genes whose expression either increased or decreased in two of the three parous/nulliparous sample sets, that also had a confidence level > 90% (Fig. 2). Overall, the true-positive rate ascertained to date is approximately 70%. This true-positive rate is considerably higher than that from any of the procedures that we have previously used to screen for differentially expressed genes. Approximately 80 genes were tested by Northern hybridization.

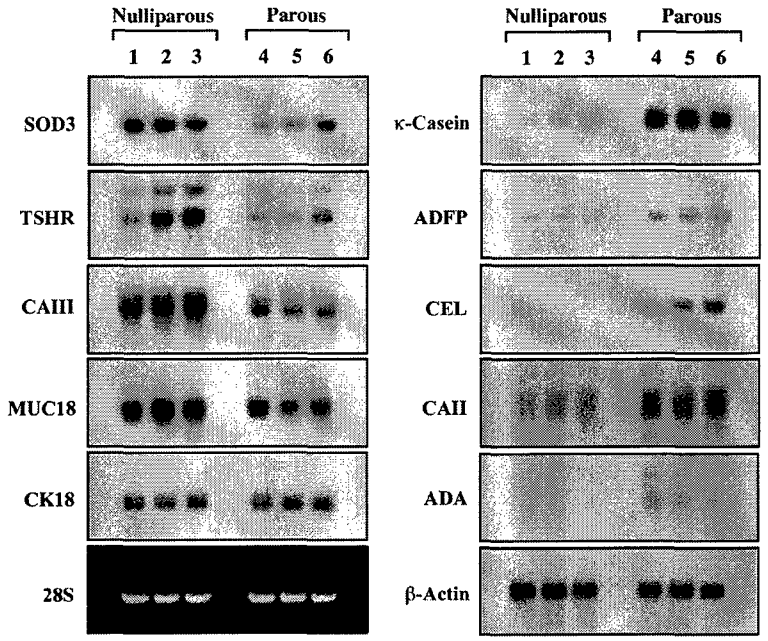

Figure 2: Differential expression of genes isolated by oligonucleotide microarrays

Since the phenomenon of parity-induced protection against breast cancer is conserved among humans, rats, and mice, we predicted that the molecular changes that underlie this effect would be conserved. As described above, microarray analysis of gene expression profiles in the mammary glands of parous and nulliparous FVB mice, Balb/c mice, and Lewis rats indicated that parity-dependent changes in gene expression are largely conserved in different strains of mice and rats. To extend these findings, we performed Northern analysis on pools of mammary gland total RNA isolated from nulliparous and parous 129SvEv and Balb/c mice, as well as from nulliparous and parous Sprague-Dawley rats, another widely used model for parity-induced protection against breast cancer. We found that all ten genes examined in 129SvEv and Balb/c mouse strains were differentially expressed in a manner similar to that observed in FVB mice (Fig. 3B and data not shown). Likewise, each of the nine genes examined in the rat exhibited a parity-dependent differential pattern of expression identical to that observed in the mouse (Fig. 3C and data not shown). When taken together with the above microarray data, these findings indicate that many of the parity-related molecular changes identified in this study are conserved among species that display parity-induced protection against mammary tumorigenesis. (Fig. 3).

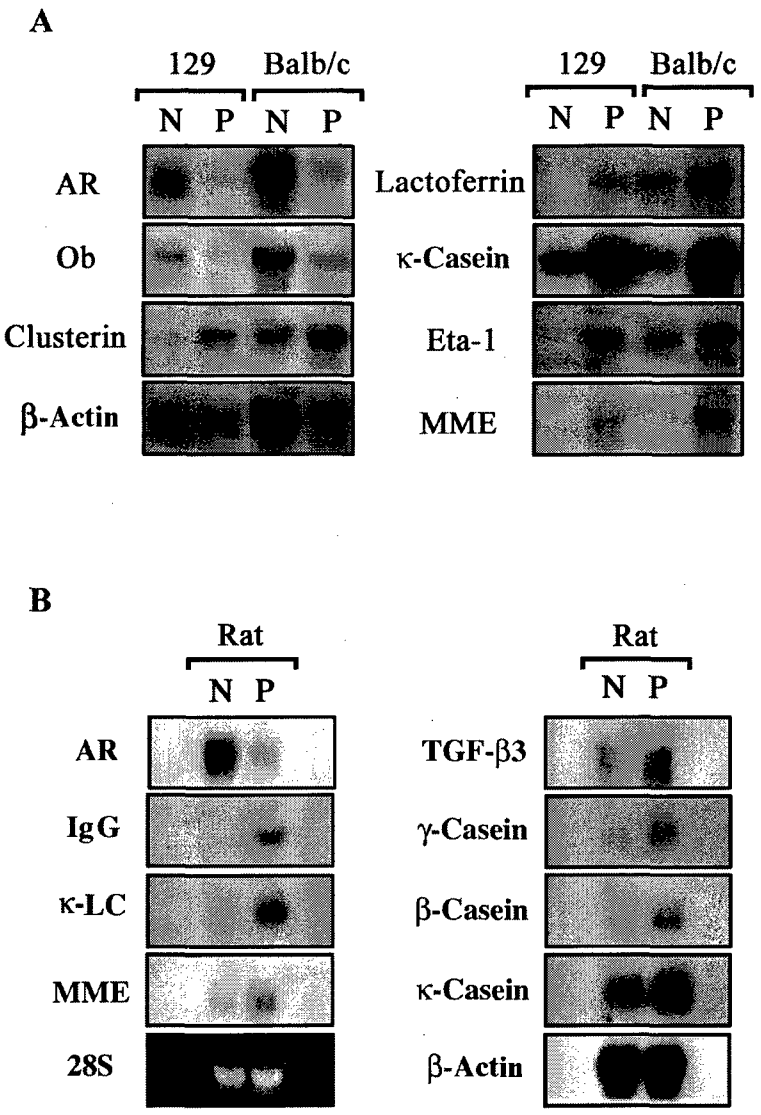

Figure 3: Differential expression of selected genes in mice and rats

Technical Objective II: Analyze genes expressed in a developmental stage-specific manner.

Task 1: *Months 6-24: Determine the developmental expression pattern of cDNA markers.*

This task has been completed on schedule. We have used the genes that we have identified as being differentially expressed to define parity-induced cellular and molecular changes that occur in the breastby examining the developmental expression pattern for those genes that we have confirmed as being differentially expressed as a function of parity. Gene expression patterns have been determined during mammary gland development using Northern hybridization. Developmental time points analyzed included virgin development from a point preceding puberty through the completion of ductal morphogenesis (2 , 5 , 10 , and 15 weeks); early, mid and late pregnancy (day 7, 14, and 21); mid lactation (day 9), and early, mid and late postlactational regression (day 2, 7, and 28). For complete evaluation, see accompanying manuscript. As examples, the developmental expression of the epidermal growth factor, amphiregulin, and the heparin-binding mitogen, pleiotrophin, are shown in Figure 4. In adult animals, both amphiregulin and pleiotrophin are expressed in a nulliparous-specific manner.

Developmentally, expression of each of these genes appeared to be induced during puberty and to remain elevated thereafter in the virgin gland. Interestingly, expression of both genes subsequently decreases during mid-pregnancy and remains

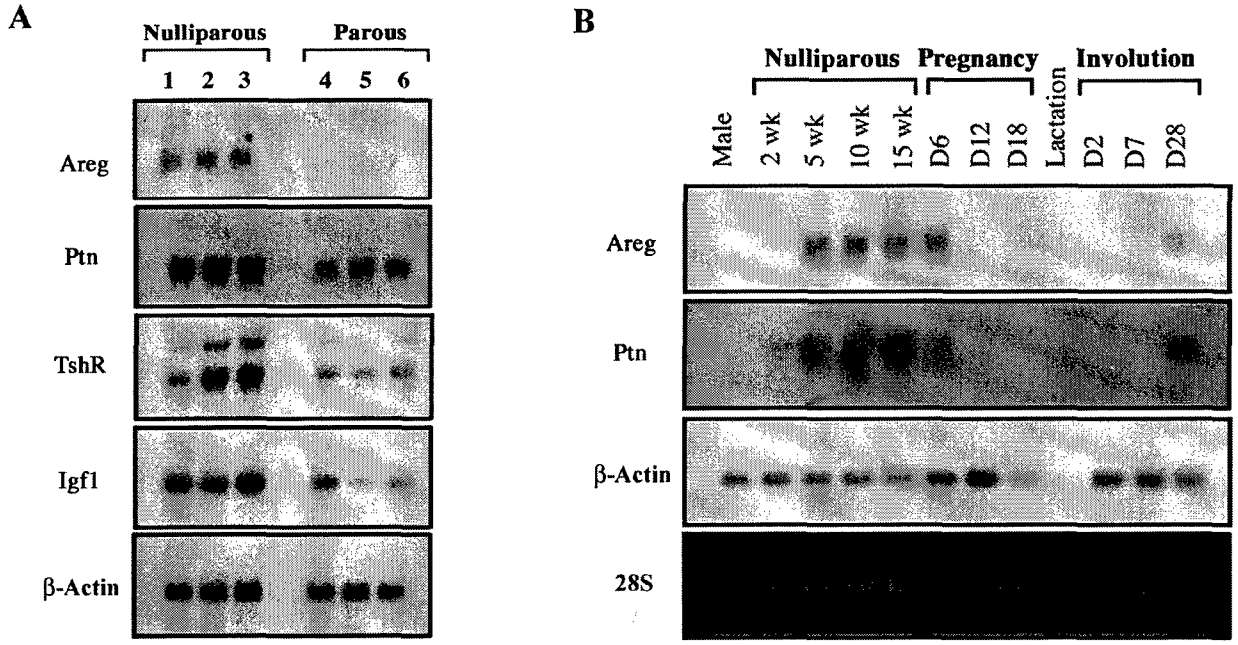

Fig. 4: Down-regulation of amphiregulin and pleiotrophin expression as a function of parity

suppressed during lactation and postlactational regression.

The developmental expression patterns of numerous other genes identified in this screen have also been examined. For example, a second category of genes whose expression was elevated in the parous involuted mammary gland included those that are specifically expressed in hematopoietic cells such as B-lymphocytes (*kappa light chain* and the *IgG, IgM,* and *IgA* heavy chains), T-lymphocytes (*T-cell death associated gene*), and macrophages (*macrophage metalloelastase* and *macrophage expressed Lipocalin-2*, that are either expressed by hematopoietic cells or are chemoattractants for these cell types

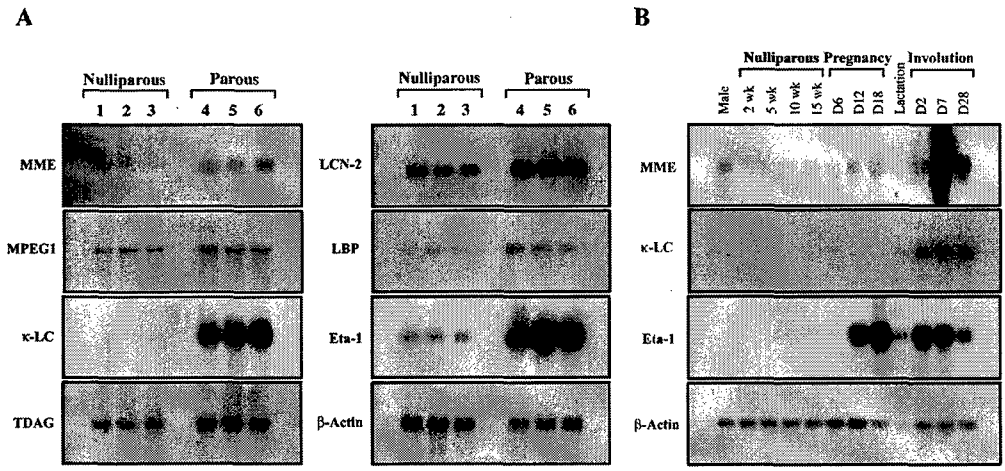

Fig. 5: Up-regulation of hematopoietic cell gene expression as a function of parity

gene 1). Additional genes, including *Early T-cell activation protein* (*Eta-1*), *LPS-Binding protein* and

were also persistently upregulated as a consequence of parity. The marked upregulation of these genes in the parous gland suggests that cells of B-lymphocyte, T-lymphocyte, and macrophage lineages may be more abundant in the parous mammary gland. To investigate this hypothesis further, we examined the expression patterns for several immune-related genes during stages of postnatal mammary development representing puberty, pregnancy, lactation and involution. Northern analysis revealed that expression of the B-cell-specific gene, *κ-light chain* (*κ-LC*), is first detected during lactation with elevated levels of expression persisting throughout postlactational involution (Fig. 5). *Macrophage metalloelastase* (*MME* or *MMP-12*) was also found to be persistently upregulated in the mammary gland as a consequence of parity (Fig. 5A). MME is a secreted metalloprotease that cleaves plasminogen to generate angiostatin, a potent inhibitor of endothelial cell proliferation. Northern hybridization demonstrated that *MME* expression levels increase dramatically at day 7 of postlactational involution and remain elevated compared to age-matched nulliparous controls following 28 days of involution (Fig. 5). Finally, we examined the developmental basis for the persistent parity-dependent upregulation of *Eta-1* expression. Northern analysis revealed that *Eta-1* expression, which has been reported in macrophages and T-lymphocytes as well as in mammary epithelial cells, is dramatically upregulated at day 12 of pregnancy and remains high through day 7 of involution (Fig. 5). Though declining somewhat by day 28 of postlactational involution, *Eta-1* expression remains markedly elevated compared to age-matched nulliparous animals. In aggregate, our data suggest that parity induces persistent increases in populations of hematopoietic cells present within the mammary gland, as well as changes in cytokine expression within the mammary epithelium itself.

Task 2: *Months 6-24: Determine the spatial pattern of expression of cDNA markers as a function of reproductive endocrine history.*

This task has been completed on schedule. The parity-specific expression of a given gene could reflect a global increase in its expression in all expressing cells, an increase in the percentage of expressing cells in the breast, or both. In order to distinguish between these possibilities, we are performing in situ hybridization for those genes that have been identified as being differentially expressed as a function of parity. As an example, the spatial expression of

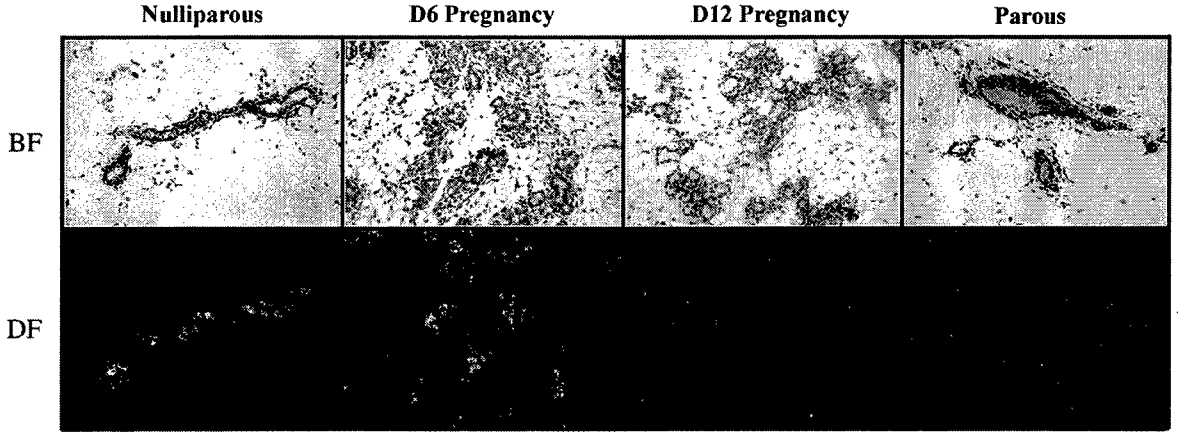

Fig. 6: Spatial expression of amphiregulin during development

amphiregulin is shown during development (Fig. 6). This analysis demonstrates that the expression of amphiregulin decreases in each expressing cell, and also that the number of amphiregulin-expressing cells decreases as a result of parity.

Similar approaches have been applied to the other differentially expressed genes isolated in this panel. For example, analysis of the spatio-temporal pattern of gene expression for the hematopoietic markers described above has also been determined (Fig. 7). These finding clearly demonstrate that

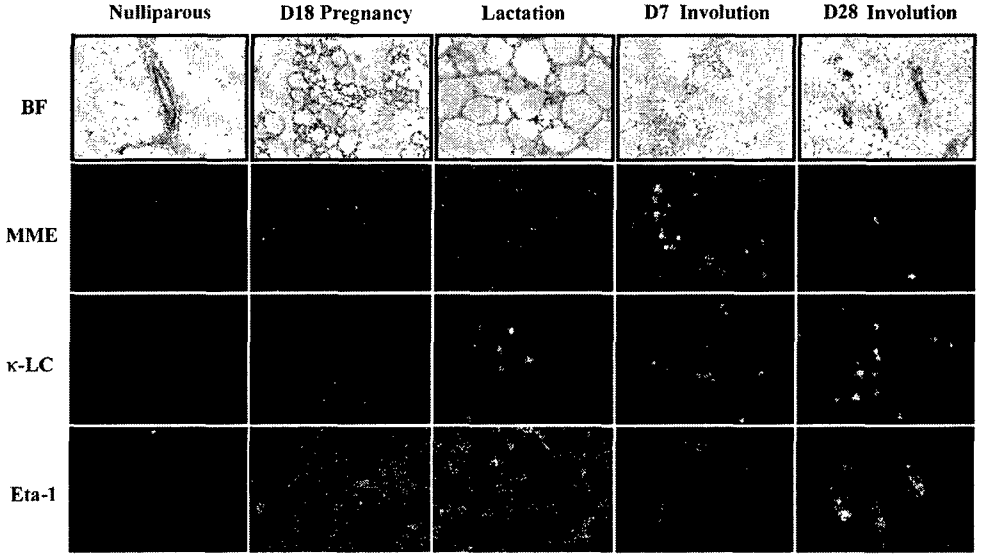

Fig. 7: Spatial expression of hematopoietic gene expression during development

parity-dependent increases in expression of MME, *κ-LC* and Eta-1 are due to increases in the number of cells that express these genes.

Task 3: *Months 6-24: Determine the relationship between the expression of cDNA markers and reproductive endocrine variables known to affect breast cancer risk.*

This task has been completed on schedule. We have determined how specific parity-related reproductive variables alter the expression of selected genes in the murine breast that we have identified as being differentially expressed as a function of parity. For example, epidemiologic findings suggest that those parity-induced changes in the mammary gland that are responsible for protection against breast cancer are likely to be permanent. Accordingly, changes in gene expression that are involved in this protective effect would be predicted to persist for periods of involution greater than 4 weeks. To determine whether the parity-dependent changes in gene expression identified in this study persist for longer period of involution, we analyzed cohorts of mice that were mated at 4 weeks of age, and then underwent 21 days of lactation and either 4, 16, or 30 weeks of postlactational involution. Northern analysis revealed that expression levels of *lactoferrin*, *κ-LC*, *TGF-β3*, *clusterin*, and *Eta-1*, were all consistently upregulated in the mammary glands of parous animals compared to age-matched nulliparous controls for up to 30 weeks of postlactational involution (Fig. 8). These findings indicate that for at least a subset of the genes identified in this study parity-induced changes in gene expression are essentially permanent. In addition, we have assessed the impact of time of first full-term pregnancy, multiparity, duration of lactation, and age, on the expression of the genes isolated in these studies.

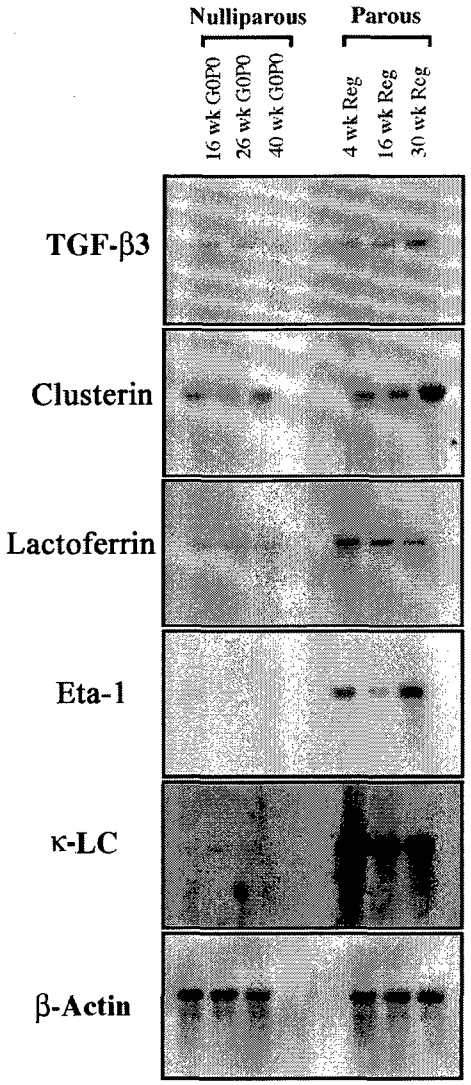

Fig. 8: Expression of parity-specific genes as a function of duration of postlactational regression

KEY RESEARCH ACCOMPLISHMENTS

- Identification of genes that are down-regulated as a function of parity.
- Identification of genes that are up-regulated as a function of parity.
- Analysis of developmental and spatial patterns of gene expression for genes regulated by parity.
- Successful application of oligonucleotide microarray technology to gene expression in whole organs.
- Validation of the overall approach proposed in this application.

REPORTABLE OUTCOMES

- A manuscript describing the results of this study has been submitted for publication. This manuscript is included in the Appendix to this report.

CONCLUSIONS

Each of the technical objectives and tasks proposed in this project have been completed on schedule. Our principal advance has been the identification of molecular changes that occur in the mammary gland as a consequence of parity. Such genetic changes have not previously been identified. We have used high-density oligonucleotide microarrays to analyze the impact of early first full-term pregnancy on global gene expression profiles within the murine mammary gland. This approach has led to the identification of a panel of genes whose expression in the mammary gland is persistently altered as a consequence of parity in multiple strains of mice as well as in two widely used rat models for parity-induced protection against breast cancer. Our findings demonstrate that parity induces the persistent downregulation of multiple genes encoding epithelial growth factors as well as the persistent upregulation of the growth-inhibitory molecule, *TGF-β3*, and several of its downstream targets. Our findings further indicate that parity results in a persistent increase in the differentiated state of the mammary gland as well as permanent changes in the hematopoietic cell types resident within the gland. These findings define a developmental state of the mammary gland that is refractory to carcinogenesis and suggest novel hypotheses for mechanisms by which parity may modulate breast cancer risk. We believe that these studies represent an important step towards determining the mechanisms by which breast cancer susceptibility is modulated by reproductive history. This is particularly important since the identification and use of intermediate molecular endpoints that accurately identify changes in the breast associated with changes in breast cancer risk will ultimately facilitate the development of chemopreventative regimens that mimic parity-induced protection against breast cancer.

REFERENCES

1. Russo, J., R. Rivera, and I.H. Russo, *Influence of age and parity on the development of the human breast.* Breast Cancer Research & Treatment, 1992. **23**(3): p. 211-8.
2. de Saizieu, A., *et al.*, *Bacterial transcript imaging by hybridization of total RNA to oligonucleotide arrays.* Nature Biotechnology, 1998. **16**: p. 45-48.
3. Wodicka, L., *et al.*, *Genome-wide expression monitoring in Saccharomyces cerevisiae.* Nature Biotechnology, 1997. **15**: p. 1359-1367.
4. Lockhart, D., *et al.*, *Expression monitoring by hybridization to high-density oligonucleotide arrays.* Nature Biotechnology, 1996. **14**: p. 1675-1680.

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Early First Full-Term Pregnancy Results in Permanent Changes in Gene Expression in the Murine Mammary Gland

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ABSTRACT

Epidemiologic studies have repeatedly demonstrated that women who undergo a first full-term pregnancy early in life have a significantly reduced lifetime risk of breast cancer. Similar to humans, rodents that have previously undergone a full-term pregnancy are highly resistant to carcinogen-induced breast cancer compared to age-matched nulliparous controls. Unfortunately, little progress has been made over the past 30 years towards understanding the cellular and molecular basis of parity-induced protection against breast cancer. We have used high-density DNA oligonucleotide microarrays to identify genes whose expression in the murine mammary gland is persistently altered as a consequence of an early first full-term pregnancy. The panel of differentially expressed genes that we have isolated in FVB mice reproducibly distinguishes between the nulliparous and parous states of the mammary gland in multiple strains of mice and rats. We find that parity results in the persistent down-regulation of multiple genes encoding epithelial growth factors as well as the persistent upregulation of the growth-inhibitory molecule, *TGF-ß3*, and several of its transcriptional targets. Our studies further indicate that parity results in a persistent increase in the differentiated state of the mammary gland as well as persistent changes in the immunologic environment and hematopoietic cell types resident within the gland. These data provide the first molecular description of a developmental state of the mammary gland that is associated with reduced cancer risk and suggest novel hypotheses for the mechanistic basis by which parity may modulate breast cancer risk.

INTRODUCTION

Numerous epidemiologic studies have shown that women who undergo a full-term pregnancy early in life have a significantly reduced lifetime risk of breast cancer (1-10). In contrast, women who undergo their first full-term pregnancy after age 30 have a risk of breast cancer that is actually higher than that of nulliparous women. This suggests that parity-induced protection against breast cancer is principally dependent upon the *timing* of a first full-term pregnancy, rather than on its occurrence *per se*. Other reproductive variables such as multiparity and lactation have also been shown to reduce breast cancer risk, but in a manner that is independent of age at first childbirth. Moreover, these effects are generally modest in comparison to age at first full-term pregnancy (1, 6, 11-16).

Interestingly, women from different countries and ethnic groups exhibit a similar degree of parity-induced protection against breast cancer regardless of whether the regional incidence of this malignancy is high, as in Western countries, or low as in the Far East. This suggests that the reduction in breast cancer risk associated with early first full-term pregnancy does not result from extrinsic factors specific to a particular environmental, genetic, or socioeconomic setting, but rather from an intrinsic effect of parity on the biology of the breast. In principle, this protective effect could result from the pregnancy-driven terminal differentiation of a subpopulation of target cells at increased risk for carcinogenesis, from the preferential loss of target cells during postlactational involution, or from a permanent endocrine change that indirectly decreases breast cancer risk by altering either the hormonal environment or the hormonal responsiveness of cells in the mammary gland [Russo, 1978 #1181; Russo, 1979 #719; Russo, 1982 #1182; Thordarson, 1995 #1748; Swanson, 1995 #1739; (17-19)]. To date, however, little evidence exists to support of any of these hypotheses at the cellular or molecular

level. As such, more than 30 years after the landmark description of this phenomenon, the mechanisms that underlie parity-induced protection against breast cancer remain largely unknown.

Like humans, both rats and mice exhibit parity-induced protection against breast cancer. For example, administration of the carcinogens 7,12-dimethylbenz(a)anthracene or N-methylnitrosourea to nulliparous rats induces mammary adenocarcinomas that are hormone-dependent and histologically similar to human breast tumors [Russo, 1978 #1181; Russo, 1979 #719; Russo, 1977 #339; Moon, 1969 #261; Moon, 1981 #262; Huggins, 1959 #174; Huggins, 1959 #170; (20-22)Guzman, 1999 #1558; (23)Yang, 1999 #1786]. In contrast, rats that have previously undergone a full-term pregnancy are highly resistant to the induction of breast cancer by carcinogen administration. Similar to rats, mice that have undergone an early first full-term pregnancy have also been shown to be less susceptible to carcinogen-induced mammary tumors than age-matched nulliparous controls (24). These studies indicate that key epidemiological features of the influence of reproductive history on breast cancer risk in humans are mirrored in rodent model systems. The use of animal models to study parity-induced protection against breast cancer is further facilitated by the many similarities in structure, function, and development that exist between the human and rodent mammary gland (25, 26). Since the mechanisms underlying parity-induced changes in breast cancer risk are likely to involve complex genetic and epigenetic events, animal models that recapitulate epidemiological findings, permit critical aspects of reproductive history to be rigorously controlled, reduce genetic variation, and permit the examination of molecular and cellular events at defined developmental stages of interest in normal tissue will be critical for understanding this phenomenon.

Despite long-standing evidence for the differential susceptibility of the parous and

nulliparous breast to carcinogenesis, a comprehensive analysis of the molecular and cellular changes induced in the breast by parity has not been previously reported. Such information would not only define a protected state of the mammary gland at the molecular level, but could also provide insight into the pathways that underlie parity-induced protection. In addition, identifying a panel of molecular markers whose expression is reproducibly altered by early first full-term pregnancy would provide candidate intermediate molecular endpoints by which to monitor the efficacy of pharmacological interventions designed to mimic this naturally occurring protective event.

In this report we have used high-density oligonucleotide microarrays to analyze the impact of early first full-term pregnancy on global gene expression profiles within the murine mammary gland. This approach has led to the identification of a panel of genes whose expression in the mammary gland is persistently altered as a consequence of parity in multiple strains of mice as well as in two widely used rat models for parity-induced protection against breast cancer. Our findings demonstrate that parity induces the persistent downregulation of multiple genes encoding epithelial growth factors as well as the persistent upregulation of the growth-inhibitory molecule, *TGF-ß3*, and several of its downstream targets. Our findings further indicate that parity results in a persistent increase in the differentiated state of the mammary gland as well as permanent changes in the hematopoietic cell types resident within the gland. These findings define a developmental state of the mammary gland that is refractory to carcinogenesis and suggest novel hypotheses for mechanisms by which parity may modulate breast cancer risk.

RESULTS

Parity Results in Permanent Morphological Changes in the Mammary Gland

In humans, parity-induced changes in breast cancer susceptibility are accompanied by morphological alterations in the mammary gland (26-28). These structural changes persist throughout life and have been interpreted as a parity-induced increase in the differentiated state of the breast (26-28). Parity-induced changes in morphology and cancer susceptibility have also been observed the mammary glands of rats and mice [Sinha, 1988 #1903; Sivaraman, 1998 #1724; Russo, 1980 #1703; Moon, 1969 #261(22, 24)]. To extend these findings, we compared the morphology of mammary glands from nulliparous and parous Sprague-Dawley rats, a widely used model for parity-induced protection against breast cancer, with those from C57Bl/6 and FVB mice.

Following mating at an age corresponding to the onset of puberty, rats and mice were allowed to undergo a single round of pregnancy, 21 days of lactation, and 28 days of postlactational involution. Examination of carmine-stained whole mounts prepared from these animals demonstrated that the epithelial trees of both the rat and mouse parous involuted mammary gland are more highly branched than those of age-matched nulliparous littermates (Fig. 1). These differences persist for at least 30 weeks of postlactational involution indicating that the morphological changes induced by parity are essentially permanent (data not shown). Despite the marked differences in ductal branching patterns between nulliparous and parous mammary glands, hematoxylin and eosin-stained sections reveal that the relative amount of epithelium contained within the mammary fat pad is similar in both developmental states (Fig. 1 and data not shown). Thus, architectural differences between the nulliparous and parous mouse mammary gland are easily distinguishable, occur in multiple strains, are conserved among rodent

species, and are analogous to those that have been described in the human breast (26, 27).

Microarray Analysis of Parity-Induced Changes in Gene Expression

Since parity-induced changes in both the structure of the mammary gland and its susceptibility to cancer are conserved between humans and rodents, we sought to define the molecular differences between the nulliparous and parous mammary gland using high density oligonucleotide microarrays. These studies were intended to define a developmentally protected state of the mammary gland at the molecular level as well as facilitate the identification of parity-induced changes in the abundance of different cell types within the mammary gland. While we anticipated that many of the differentially expressed genes identified by this approach might not play a causal role in parity-induced protection, we nevertheless considered it likely that differentially expressed genes would provide insight into parity-induced alterations in the mammary gland, including those that are responsible for the resistance of the parous gland to carcinogenesis.

Oligonucleotide microarray expression profiling was performed in triplicate on pooled mammary gland samples, each of which was derived from 15-20 animals to control for sources of biological variation (29). Since stromal-epithelial interactions have been clearly shown to affect the behavior of mammary epithelial cells, expression changes were profiled in intact mammary glands with the exception that the lymph node present in the number 4 mammary gland was removed [Wiesen, 1999 #1775(30-33)]. Although the morphological changes characteristic of postlactational involution are essentially complete after 14 days, we chose to profile parous animals after 28 days of involution to facilitate the identification of persistent changes in gene expression due to parity rather than acute changes in gene expression due to the processes of pregnancy, lactation or involution per se.

Three independent, age-matched pools of total RNA derived from nulliparous (15 wk G0P0) and parous (15 wk G1P1) cohorts were hybridized to high-density oligonucleotide microarrays representing approximately 5,300 murine genes and ESTs (Mu6500). Affymetrix comparative algorithms were used to identify genes whose expression levels consistently changed as a consequence of parity. Northern hybridization analysis performed on independent nulliparous and parous mammary gland samples confirmed the differential expression of 14/14 genes identified by Affymetrix algorithms as being differentially expressed in each of the three independent microarray comparisons (Fig. 2 and data not shown). This approach, combined with Northern analysis of candidate genes identified by microarray analysis as being differentially expressed in two out of three sets of nulliparous and parous samples, resulted in the identification of 41 differentially expressed genes (Table 1 and Fig. 2). These include 10 genes that are preferentially expressed in the nulliparous mammary gland as well as 31 genes that are preferentially expressed in the parous mammary gland. Notably, expression levels of the epithelial cell marker, *cytokeratin18*, do not differ between the nulliparous and parous mammary gland, indicating that the observed changes in gene expression are not merely a consequence of the expansion or contraction of the epithelial cell compartment (Fig. 2).

Differentially Expressed Genes Distinguish Parous and Nulliparous Tissues in Mice and Rats

Having identified a panel of genes that were differentially expressed in a parity-dependent manner in an index group of FVB mice, we asked whether the expression patterns of these genes were sufficient to consistently distinguish nulliparous and parous mammary tissues harvested from independent groups of FVB mice as well as from additional strains of mice. To

this end, we generated 12 additional independent sets of pooled mammary gland RNA samples from parous and nulliparous FVB and Balb/c mice. These samples were subjected to high-density oligonucleotide microarray analysis on MGU74A mouse arrays and clustered based on expression profiles for the differentially expressed genes identified in our original Mu6500 array analysis. Cluster analysis performed in a blinded manner demonstrated that the expression patterns for the 41 genes identified in this study were sufficient to correctly distinguish nulliparous and parous mammary gland samples harvested from independent sets of FVB mice (Fig. 3A). Furthermore, the expression patterns for these same genes were also sufficient to correctly distinguish nulliparous and parous mammary gland samples harvested from Balb/c mice (Fig. 3B). Given that different generations of microarrays composed of different probe sets were used to perform these analyses, our findings suggest that this panel of genes accurately and reproducibly identifies parity-induced changes in the mouse mammary gland (Fig. 3A).

Since Sprague-Dawley and Lewis rats represent the most widely used models of parity-induced protection against breast cancer, we were further interested in determining whether genes identified as having a parity-dependent pattern of expression in FVB mice would be sufficient to predict correctly the reproductive histories of rats. As such, 6 independent sets of pooled mammary gland RNA samples from parous and nulliparous Lewis rats were analyzed on RGU34A high-density rat microarrays To facilitate comparison of mouse data sets genes to microarray expression data obtained from rats, genes identified as being expressed in parity-dependent manner in FVB mice were mapped via Homologene to the rat genome. Cluster analysis of rat microarray data revealed that the panel of genes identified on the basis of their parity-dependent expression in FVB mice was sufficient to correctly distinguish mammary gland samples harvested from nulliparous and parous Lewis rats (Fig. 3C). These findings demonstrate

that the expression patterns of the genes that we have isolated are reproducibly and persistently altered as a consequence of parity, and that these parity-induced changes in gene expression are conserved in different mouse strains and rodent species that exhibit parity-induced protection against breast cancer.

Functional Gene Categories Accurately Predict Reproductive History

Examination of the genes identified as being differentially expressed as a consequence of parity revealed several distinct functional categories (Table 1). These include: growth-promoting molecules such as amphiregulin, pleiotrophin, and insulin-like growth factor I; molecules related to epithelial differentiation such as milk proteins; molecules expressed by hematopoietic cells such as B-cells, T-cells and macrophages; and molecules involved in the TGF-ß pathway. In light of this observation, we asked whether smaller subsets of genes representing these four functional gene categories (growth factor-related: 4 genes; differentiation-related: 7 genes; immune-related: 15 genes; and TGF-ß-related: 4 genes) would be sufficient to correctly distinguish nulliparous and parous mammary gland samples in a blinded manner.

Strikingly, even when considered in isolation, gene expression patterns within each of these four subgroups were found to be sufficient to identify correctly parous and nulliparous mammary samples derived from FVB mice, Balb/c mice and Lewis rats (Fig. 3D, 3E and data not shown). These findings demonstrate that expression changes within each of these four functional gene categories robustly and independently distinguish between the parous and the nulliparous states of the mammary gland in different rodent strains and species that exhibit parity-induced protection against breast cancer. Our observations further suggest that the downregulation of specific genes involved in epithelial proliferation, and the upregulation of

genes involved in epithelial differentiation, immune regulation, and TGF-ß-mediated growth inhibition represent cardinal features of parity-induced changes in the mammary gland.

Differentiation Markers are Preferentially Expressed in the Parous Mammary Gland

A prominent category of genes whose expression was elevated in the parous involuted mammary gland included markers for mammary epithelial differentiation such as *α-casein, ß-casein, γ-casein, κ-casein, whey acidic protein, lactoferrin* and *α-lactalbumin* (Table 1). Cluster analysis of microarray data obtained for 6 pooled mammary samples harvested from independent sets of FVB mice demonstrated that the expression patterns of these seven genes were sufficient to correctly distinguish nulliparous and parous FVB samples in a blinded manner (Fig. 3 and Table 1). The differential expression patterns of these 7 genes were also sufficient to distinguish correctly nulliparous and parous mammary samples harvested from Balb/c mice as well as from Lewis rats (Fig. 3 and data not shown). Additional genes associated with epithelial differentiation, such as *connexin 26*, were also found to be persistently upregulated by parity (Table 1). These observations indicate that the parity-dependent upregulation of markers for mammary epithelial differentiation is conserved among different mouse strains and rodent species that exhibit parity-induced protection against breast cancer. These findings provide the first molecular evidence supporting the hypothesis that parity results in a persistent increase in the differentiated state of the mammary epithelium (34). In addition, the preferential expression of *ADFP*, which is upregulated in differentiated adipocytes, in the parous involuted gland suggests that stromal compartments of the mammary gland may also become more differentiated as a consequence of parity (35).

Parity-Induced Changes in Hematopoietic Cells in the Mammary Gland

A second category of genes whose expression was elevated in the parous involuted mammary gland included those that are specifically expressed in hematopoietic cells such as B-lymphocytes (*kappa light chain* and the *IgG, IgM,* and *IgA* heavy chains), T-lymphocytes (*T-cell death associated gene*), and macrophages (*macrophage metalloelastase* and *macrophage expressed gene 1*) (Table 1 and Fig. 4A). Additional genes, including *Early T-cell activation protein* (*Eta-1*), *LPS-Binding protein* and *Lipocalin-2*, that are either expressed by hematopoietic cells or are chemoattractants for these cell types were also persistently upregulated as a consequence of parity (36-39). Similar to markers of epithelial differentiation, the expression patterns of this subset of immune-related genes were able in a blinded manner to identify correctly parous and nulliparous mammary gland samples in FVB mice, Balb/c mice and Lewis rats (Fig. 3 and data not shown). The marked upregulation of these genes in the parous gland suggests that cells of B-lymphocyte, T-lymphocyte, and macrophage lineages may be more abundant in the parous mammary gland.

To investigate this hypothesis further, we examined the expression patterns for several immune-related genes during stages of postnatal mammary development representing puberty, pregnancy, lactation and involution. Northern analysis revealed that expression of the B-cell-specific gene, *κ-light chain* (*κ-LC*), is first detected during lactation with elevated levels of expression persisting throughout postlactational involution (Fig. 4B). The increase in *κ-LC* expression during lactation is partially masked by the dilutional effects that result from the massive increase in milk protein gene expression that occurs during this period, as evidenced by the apparent decrease in _-actin expression that occurs during this same period (Fig. 4B) (40, 41). In situ hybridization confirmed the upregulation of *κ-LC* expression during lactation and

further revealed that this upregulation is due to an increase in the number of *κ-LC* expressing cells (Fig. 4C and data not shown). The spatio-temporal pattern of *κ-LC* expression is consistent with the reported influx of lymphocytes into the breast that occurs during lactation (42-44). Surprisingly, however, this lymphocyte population appears to persist in the fully involuted gland.

Macrophage metalloelastase (*MME* or *MMP-12*) was also found to be persistently upregulated in the mammary gland as a consequence of parity (Fig. 4A). MME is a secreted metalloprotease that cleaves plasminogen to generate angiostatin, a potent inhibitor of endothelial cell proliferation (45-47). Northern hybridization demonstrated that *MME* expression levels increase dramatically at day 7 of postlactational involution and remain elevated compared to age-matched nulliparous controls following 28 days of involution (Fig. 4B). *In situ* hybridization revealed that *MME* expression is restricted to isolated cells within the mammary stroma at day 7 of involution (Fig. 4C and data not shown). This finding is consistent with this gene's reported expression in macrophages and with previous evidence that macrophages are recruited to the breast during involution where they participate in the clearance of post-apoptotic debris (48, 49). Interestingly, by day 28 of involution foci of *MME* expression became tightly associated with the epithelial compartment reflecting either a persistent population of macrophages residing within the parous epithelium or a subset of epithelial cells expressing *MME* (Fig. 4C).

Finally, we examined the developmental basis for the persistent parity-dependent upregulation of *Eta-1* expression (Fig. 4A). Northern analysis revealed that *Eta-1* expression, which has been reported in macrophages and T-lymphocytes as well as in mammary epithelial cells, is dramatically upregulated at day 12 of pregnancy and remains high through day 7 of involution (Fig. 4B) (50, 51). Though declining somewhat by day 28 of postlactational

involution, *Eta-1* expression remains markedly elevated compared to age-matched nulliparous animals. Notably, *in situ* hybridization analysis demonstrated that *Eta-1* expression in the parous involuted gland is restricted to a subset of mammary epithelial cells (Fig. 4C and data not shown). In aggregate, our data suggest that parity induces persistent increases in populations of hematopoietic cells present within the mammary gland, as well as changes in cytokine expression within the mammary epithelium itself.

Parity Results in a Decrease in Growth Factor Expression

Interestingly, genes that encode growth regulatory molecules constitutex more than half of genes that were found to be persistently downregulated by parity (Table 1). These include *amphiregulin (Areg), pleiotrophin (Ptn), insulin-like growth factor* 1 *(Igf1), leptin (Ob),* and *thyroid stimulating hormone receptor (TshR)* (Table 1 and Fig. 5A). Cluster analysis further demonstrated that the expression patterns of these 5 genes were sufficient in a blinded manner to identify correctly parous and nulliparous mammary samples in FVB mice, Balb/c mice and Lewis rats (Fig. 3). This indicates that downregulation of the genes encoding these growth factors is a characteristic feature of parity-induced changes in the rodent mammary gland.

We investigated the developmental expression pattern of the epidermal growth factor receptor ligand, *Areg,* and the heparin-binding mitogen, *Ptn*, to determine the basis for their preferential expression in the nulliparous gland. Strikingly, the temporal patterns of expression for these molecules are virtually identical during postnatal mammary development (Fig. 5B). Steady state levels of *Areg* and *Ptn* mRNA are dramatically upregulated in the female mammary gland between 2 weeks and 5 weeks of age, a period corresponding to the onset of ductal morphogenesis **(David Lee ref)**. Levels of *Areg* and *Ptn* expression remain relatively constant

throughout nulliparous development and early pregnancy, then decrease sharply by mid-pregnancy (day 12) and remain low throughout lactation and involution (Fig. 5B). I*n situ* hybridization analysis confirmed the pregnancy-induced downregulation of *Areg* expression as well as the persistent downregulation of *Areg* expression throughout the epithelial compartment of the parous gland (Fig. 5C). Together with the observed decreases in *Ptn* and *Igf1* expression levels, our data suggest the possibility that parity results in the downregulation of multiple pathways that stimulate epithelial proliferation.

Parity Results in Increased Mammary Expression of TGF-ß3 and its Targets

Our microarray data indicated that steady-state mRNA levels for the growth-inhibitory cytokine, *TGF-ß3,* were persistently upregulated in the mammary glands of FVB mice as a consequence of parity (Table 1, Fig. 4, and Fig. 6). In addition, *clusterin*, *Eta-1* and *Id-2*, each of which has previously been implicated as a downstream transcriptional target of *TGF-ß3,* were also found to be persistently upregulated by parity (49, 52-56). Cluster analysis demonstrated that the expression patterns of these four genes are sufficient to correctly distinguish parous and nulliparous mammary samples in FVB mice, Balb/c mice and Lewis rats. Northern and *in situ* hybridization analysis confirmed the parity-dependent upregulation of *TGF-ß3*, *clusterin*, and *Eta-1* expression in the parous mammary gland, and further demonstrated that these genes are primarily expressed in the epithelial compartment (Fig. 6A and 6C; Fig. 4A and 4C). Northern analysis of the developmental expression patterns of *TGF-ß3* and *clusterin* demonstrated that both genes exhibit maximal expression at day 2 of involution with a secondary peak of expression at day 12 of pregnancy (Fig. 6B and 6C). These developmental expression profiles are consistent with a role for these molecules in cell death during mammary gland involution (49,

53, 57). The coordinate elevation in expression of *TGF-ß3* along with several of its transcriptional targets suggests that the upregulation of *TGF-ß3* mRNA in the parous mammary gland may be accompanied by a *bona fide* increase in *TGF-ß3* activity.

Parity-Induced Changes in Gene Expression Persist in the Mammary Gland

Epidemiologic findings suggest that those parity-induced changes in the mammary gland that are responsible for protection against breast cancer are likely to be permanent. Accordingly, changes in gene expression that are involved in this protective effect would be predicted to persist for periods of involution greater than 4 weeks. To determine whether the parity-dependent changes in gene expression identified in this study persist for longer period of involution, we analyzed cohorts of mice that were mated at 4 weeks of age, and then underwent 21 days of lactation and either 4, 16, or 30 weeks of postlactational involution. Northern analysis revealed that expression levels of *lactoferrin*, *_-LC*, *TGF-ß3*, *clusterin*, and *Eta-1*, were all consistently upregulated in the mammary glands of parous animals compared to age-matched nulliparous controls for up to 30 weeks of postlactational involution (Fig. 7A). These findings indicate that for at least a subset of the genes identified in this study parity-induced changes in gene expression are essentially permanent.

Parity-Induced Changes in Gene Expression are Conserved Across Species

Since the phenomenon of parity-induced protection against breast cancer is conserved among humans, rats, and mice, we predicted that the molecular changes that underlie this effect would be conserved. As described above, microarray analysis of gene expression profiles in the mammary glands of parous and nulliparous FVB mice, Balb/c mice, and Lewis rats indicated

that parity-dependent changes in gene expression are largely conserved in different strains of mice and rats. To extend these findings, we performed Northern analysis on pools of mammary gland total RNA isolated from nulliparous and parous 129SvEv and Balb/c mice, as well as from nulliparous and parous Sprague-Dawley rats, another widely used model for parity-induced protection against breast cancer. We found that all ten genes examined in 129SvEv and Balb/c mouse strains were differentially expressed in a manner similar to that observed in FVB mice (Fig. 7B and data not shown). Likewise, each of the nine genes examined in the rat exhibited a parity-dependent differential pattern of expression identical to that observed in the mouse (Fig. 7C and data not shown). When taken together with the above microarray data, these findings indicate that many of the parity-related molecular changes identified in this study are conserved among species that display parity-induced protection against mammary tumorigenesis.

DISCUSSION

We have used DNA oligonucleotide microarrays to analyze the expression of ~5,300 genes and ESTs in order to identify persistent changes in gene expression in the murine mammary gland that are induced by an early first full-term pregnancy. Using this approach, we have isolated a panel of genes whose expression is persistently altered by a reproductive event known to reduce breast cancer risk. The expression patterns of the genes isolated reproducibly distinguish between the nulliparous and parous mammary gland in both rats and mice, as well as identify changes in the abundance of specific cell types that occur in the mammary gland as a result of parity. Our findings demonstrate at the molecular level that parity results in a persistent increase in the differentiated state of the mammary epithelium, as has been previously suggested based on morphological criteria. In addition, our data suggest several new hypotheses for the mechanistic basis of parity-induced protection against breast cancer, including that parity may decrease the susceptibility of the mammary epithelium to malignant transformation by downregulating multiple growth promoting pathways, upregulating growth-inhibitory pathways, and/or changing the immune environment of the breast.

For the majority of genes identified in this study, parity-induced changes in gene expression were shown to be independent of the length of postlactational involution, demonstrating that the altered patterns of expression that we have identified are persistent, if not permanent. Furthermore, we have confirmed that parity-induced changes in gene expression are largely conserved in multiple stains of mice as well as in two well-characterized rat models for parity-induced protection against breast cancer. Beyond indicating that many of the molecular changes that we have defined are conserved among rodents, our findings suggest that these changes may also be relevant to parity-induced changes in the human breast.

One of the most striking findings of our microarray analysis was the parity-dependent down-regulation of multiple genes involved in the regulation of cell growth and proliferation. Specifically, the reduced expression of *Areg, Ptn, Igf1, TshR, and Ob* suggest that multiple mitogenic pathways may be downregulated in the mammary gland as a consequence of parity. Notably, elevated expression of *Areg, Ptn* and *Igf1* have each been implicated in the pathogenesis of human breast cancer (58). AREG, a ligand for the EGFR, has been shown to be overexpressed in 35-50% of primary human breast cancers (59-61). Consistent with this, *Areg* is overexpressed in hyperplastic stages of mammary tumor development in MMTV-PyMT and MT-TGFα transgenic mice (62), and is a potent stimulator of anchorage-dependent growth in nontransformed MCF-10 cells (63). Moreover, targeted inactivation of *Areg* in mice causes a marked delay in ductal elongation during puberty and inhibition of *Areg* expression in transformed mammary epithelial cell lines by antisense approaches results in growth inhibition and reduced tumorigenicity (64-66). These findings indicate that Areg plays an important role in promoting mammary epithelial cell proliferation and predict that the parity-dependent downregulation of *Areg* could contribute to a decrease in the susceptibility of the parous mammary gland to cancer.

The heparin-binding growth factor, pleiotrophin, was also found to be down-regulated in the parous mammary gland. Pleiotrophin has been implicated in angiogenesis and mammary tumor progression and a majority of primary human breast tumors display high levels of *PTN* mRNA expression, as do carcinogen-induced rat mammary tumors (67, 68). Moreover, Ptn overexpression in NIH 3T3 cells induces transformation and confers the ability to form tumors in nude mice (69). Conversely, overexpression of a dominant negative PTN mutant reverses the transformed phenotype of the human breast cancer cell line MDA-MB-231 (70). PTN has also

been reported to stimulate endothelial cell growth, migration, and tube formation. Furthermorer, tumors formed from *PTN*-transfected MCF-7 breast carcinoma cells injected into mice exhibit enhanced growth, increased endothelial proliferation, and increased vascular density compared to control cells (71-73). As such, like *Areg* the downregulation of *Ptn* represents a biologically plausible mechanism that could contribute to parity-induced protection against breast cancer.

It has previously been reported that parity results in the downregulation of the EGF receptor and growth hormone receptor in rats (22). Our finding that the EFGR ligand, Areg, and an EST that is homologous to the growth hormone receptor are downregulated by parity in the mouse provide additional support for this observation. However, our finding that *Igf1, Ptn, Ob* and *TshR*, are also downregulated by parity raises the possibility that other growth promoting pathways whose expression has not previously been correlated with reproductive history may also contribute to parity-induced protection against breast cancer. This hypothesis is particularly intriguing in light of recent findings that a strong positive correlation exists between circulating IGF1 concentrations and breast cancer risk among premenopausal women (74). As such, our studies suggest that parity may result in changes in both systemic and local growth signaling pathways in the mammary gland that may ultimately contribute to the establishment of a less mitogenic environment.

In addition to decreases in growth factor gene expression, we have also identified a parity-dependent increase in the expression of *TGF-ß3* and several of its downstream transcriptional targets in the mammary gland. The well-described role of *TGF-ß3* in growth inhibition makes the upregulation of this pathway a biologically plausible contributing factor to parity-induced protection against breast cancer. While our data do not directly demonstrate activation of the *TGF-ß3* signaling pathway in the parous mammary gland, the coordinate

regulation of downstream transcriptional targets of *TGF-β3* lend support this model. Further investigation of the activation of additional downstream molecules in this pathway, such as SMAD 2, SMAD 3 and SMAD 4 would provide additional evidence for the upregulation of this pathway.

It has previously been proposed that parity-induced protection against breast cancer may be mediated by an increased state of differentiation of the parous mammary gland (**Russo**). Our data provide the first molecular evidence to support the contention that the epithelial compartment of the parous mammary gland is more differentiated than that of the nulliparous gland, as well as evidence to suggest that parity may also increase the differentiated state of the stromal compartment of the mammary gland. However, the recent finding in rats that the dopamine antagonist, perphenazine, induces epithelial differentiation yet does not protect against carcinogen-induced mammary tumorigenesis casts doubt on a central role for differentiation in parity-induced protection against breast cancer (18). As such, while a persistent parity-induced persistent increase in the differentiated state of the mammary gland remains a biologically plausible mechanism for reducing cancer susceptibility, this finding may nevertheless be unrelated to the mechanism by which parity reduces breast cancer risk. However, since the extent of epithelial differentiation induced by perphenazine may differ from that induced by parity, further studies will be required to conclusively rule out differentiation as a contributing factor.

The finding that the secreted anti-angiogenesis factor, *MME*, is highly expressed by a subset of epithelial cells in the involuted parous mammary gland further supports the hypothesis that parity induces changes in the mammary environment that make it less hospitable for tumor establishment and progression than the nulliparous gland. The cleavage of plasminogen by

MME produces angiostatin, which potently inhibits endothelial cell proliferation *in vivo* (45-47). Gene transfer of MME into murine melanoma cells inhibits angiogenesis as well as primary tumor growth (75). Consistent with this mechanism of action, *MME* expression in human hepatocellular carcinomas correlates highly with both angiostatin protein levels and patient survival (76). Thus, upregulation of *MME* expression in the parous mammary gland may suppress the neovascularization that is required for tumor growth.

Related to changes in *MME* expression, in the course of the above experiments we were surprised to find the parity-induced upregulation of genes whose expression marks distinct classes of hematopoietic cells. These observations suggest that parity results in a persistent increase in the number of macrophages, B-lympohcytes and T-lymphocytes that reside within the mammary gland. In addition, we have shown that parity-induces changes in the expression of specific cytokines in the mammary gland, at least some of which occur within the epithelial compartment. In particular, the dramatic increase in epithelial expression of the cytokine, *Eta-1,* which has been shown to enhance migration of activated macrophages, to influence macrophage cytokine production, and to increase IgG and IgM expression in activated B cells, suggests a model for paracrine signaling mechanisms by which parity-induced changes in epithelial gene expression may influence the function of immune cells with the gland (77-79) (51). Such paracrine signaling mechanisms could represent a potential route by which parity might create an environment that is refractory to tumorigenesis. More broadly, however, our findings suggest that parity may induce widespread changes in the immunological environment of the mammary gland, potentially including tumor surveillance mechanisms, and may contribute to parity-induced protection against breast cancer.

Finally, the realization that specific reproductive endocrine events alter breast cancer risk

in a predictable fashion raises the possibility that naturally occurring events known to decrease breast cancer risk might be mimicked pharmacologically. The desire to pursue this objective is heightened by the fact that while it is now possible to identify women who are at elevated risk for developing breast cancer, few interventions currently exist. As such, reducing breast cancer risk via hormonal manipulations designed to mimic naturally occurring endocrine events could represent an attractive alternative. It is to this end that an early first full-term pregnancy has been proposed as a logical paradigm on which to model the hormonal chemoprevention of breast cancer. The achievement of this goal, however, has been hampered by our lack of understanding of the mechanisms by which reproductive events alter breast cancer risk. Understanding these mechanisms will ultimately facilitate the design of safe and effective hormonal chemoprevention regimens. Moreover, the development and testing of such regimens will be greatly facilitated by the identification and use of intermediate molecular endpoints that accurately detect changes in the breast associated with changes in breast cancer risk. We have chosen to exploit the defined relationship between parity and carcinogenesis in the breast to generate surrogate endpoint biomarkers for changes in the breast associated with a reduction in breast cancer risk. While our findings do not address whether the molecular and cellular alterations identified in this study are causally related to parity-induced protection against breast cancer, they do suggest promising new avenues for investigation. We believe that such biomarkers will ultimately prove essential for understanding the molecular and cellular basis of parity-induced protection against breast cancer, and for the rational design and testing of hormonal chemoprevention regimens aimed at mimicking this naturally occurring protective event.

EXPERIMENTAL PROCEDURES

Animals and Tissues

FVB mice and Sprague-Dawley rats were housed under barrier conditions with a 12 hr light/dark cycle and access to food and water *ad libitum*. Parous rodents were generated by mating 4-week-old mice or 9-week-old rats. Following parturition, animals were allowed to lactate for 21 days, at which time litters were weaned. Parous animals underwent 28 days of postlactational involution prior to sacrifice, at which time the #3-5 mammary glands were harvested and snap frozen, as were those of age-matched nulliparous controls. With the exception of glands used for whole mounts, the lymph nodes within gland #4 were removed.

Whole Mounts and Histology

Number 4 mammary glands were mounted on glass slides, fixed overnight in neutral buffered formalin, and transferred to 70% ethanol. For whole mounts, glands were rinsed in water for 5 min and stained in a filtered solution of 0.2% carmine (Sigma) and 0.5% aluminum potassium sulfate for 1-3 days. Glands were then dehydrated sequentially through 70%, 90%, 100% ethanol for 15 min. each, then de-fatted and stored in methyl salicylate. For histological analysis, fixed glands were blocked in paraffin, sectioned, and stained with hematoxylin and eosin.

RNA Isolation and Northern Analysis

Snap-frozen tissue was homogenized in guanidine thiocyanate supplemented with 7 µl/ml 2-mercaptoethanol, and RNA isolated by centrifugation through cesium chloride as previously

described (80). Equal amounts of RNA from each of 15-20 mice or 10 rats were combined for each independent pool. Total RNA was separated on a 1% LE agarose gel, and passively transferred to Gene Screen (NEN). Northern hybridization was performed per manufacturer's instructions using PerfectHyb Plus Hybridization Buffer (Sigma) and ^{32}P-labeled cDNA probes corresponding to Genbank sequences represented on the Affymetrix oligonucleotide microarray Mu6500 Gene Chip.

Oligonucleotide Microarray Hybridization and Analysis

Approximately 40 μg of total pooled RNA from each sample was used to generate cDNA and biotinylated cRNA as described (81). Hybridization to a set of Affymetrix Mu6500K microarrays was performed per manufacturer's instructions. Following washing and staining with streptavidin-phycoerythrin, chips were scanned using a Hewlett-Packard Gene Array Scanner. Grid alignment and raw data generation was performed using Affymetrix GeneChip 3.1 software. Raw gene expression levels were scaled and normalized data sets were compared using Affymetrix algorithms to identify differentially expressed genes.

For clustering analysis, probe sets for differentially expressed genes identified in the Mu6500 array analysis were mapped to corresponding probe sets on Affymetrix MGU74 microarrays via the Unigene and LocusLink databases. A list of orthologous probe sets on Affymetrix RGU34 chip was generated using matches obtained by querying the each differentially expressed gene identified in the mouse against the Homologene database (http://www.ncbi.nlm.nih.gov/HomoloGene/). Of the resulting matches, only curated and calculated reciprocal best match Homologene hits were selected for further analysis. Data were scaled such that the mean signal intensity was equivalent across all array samples excluding the

top and bottom 2% of data points. Samples were standardized prior to cluster analysis such that the median expression level and standard deviation for each gene equaled 0 and 1, respectively, across the set of samples being clustered. The data was then filtered to include only probe sets for genes shown to be differentially expressed between parous and nulliparous mouse samples (Table 1), or the orthologues for these genes in the rat. Cluster software was used to generate hierarchical clustering trees, which were visualized using Treeview (M. Eisen; http://www.microarrays.org/software).

In Situ Hybridization

In situ hybridization was performed as described (41). Antisense and sense probes were sythesized with the Promega *in vitro* transcription system using ^{35}S-UTP and ^{35}S-CTP from the T7 and SP6 RNA polymerase promoters of a PCR template containing the same sequences used for Northern hybridization analysis.

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REFERENCES

1. Paffenbarger, R. S. J., Kampert, J. B. & Chang, H.-G. (1980) *American Journal of Epidemiology* **112,** 258-68.
2. Negri, E., La Vecchia, C., Bruzzi, P. & al., e. (1988) *America Journal of Epidemiology* **128,** 1207-15.
3. MacMahon, B., Cole, P., Lin, T. M., Lowe, C. R., Mirra, A. P., Ravnihar, B., Salber, E. J., Valaoras, V. G. & Yuasa, S. (1970) *Bulletin of the World Health Organization* **43,** 209-21.
4. Lund, E. (1991) *Epidemiology* **2,** 285-8.
5. Ewertz, M., Duffy, S. W., Adami, H.-O., Kvale, G., Lund, E., Meirik, O. & al., e. (1990) *International Journal of Cancer* **46,** 597-603.
6. Layde, P. M., Webster, L. A., Baughman, A. L. & al., e. (1989) *Journal of Clinical Epidemiology* **42,** 963-73.
7. Bain, C., Willett, W., Rosner, B. & al., e. (1981) *American Journal of Epidemiology* **114,** 705-9.
8. Brinton, L. A., Hoover, R. & Fraumeni, J. F. J. (1983) *Br. J. Cancer* **47,** 757-62.
9. Rosner, B. & Colditz, G. A. (1996) *Journal of the National Cancer Institute* **88,** 359-364.
10. Rosner, B., Colditz, G. A. & Willett, W. C. (1994) *American Journal of Epidemiology* **139,** 819-35.
11. Yuan, J. M., Yu, M. C., Ross, R. K., Gao, Y. T. & Henderson, B. E. (1988) *Cancer Research* **48,** 1949-53.

12. Wang, Q.-S., Ross, R. K., Yu, M. C. & al., e. (1992) *Cancer Epidemiol. Biomark. Prev.* **1,** 435-9.

13. Bruzzi, P., Negri, E., La Vecchia, C., Decarli, A., Palli, D., Parazzini, F. & Rosselli Del Turco, M. (1988) *British Medical Journal* **297,** 1096-8.

14. Pathak, D. R. & Whittemore, A. S. (1992) *American Journal of Epidemiology* **135,** 153-168.

15. Kvale, G., Heuch, I. & Eide, G. E. (1987) *American Journal of Epidemiology* **126,** 831-41.

16. Vatten, L. J. & Kvinnsland, S. (1992) *European Journal of Cancer* **28A,** 1148-53.

17. Thordarson, G., Van Horn, K., Guzman, R. C., Nandi, S. & Talamantes, F. (2001) *Carcinogenesis* **22,** 1027-33.

18. Guzman, R. C., Yang, J., Rajkumar, L., Thordarson, G., Chen, X. & Nandi, S. (1999) *Proc Natl Acad Sci U S A* **96,** 2520-5.

19. Nandi, S., Guzman, R. & Yang, J. (1995) *Proceedings of the National Academy of Science USA* **92,** 3650-3657.

20. Huggins, C., Grand, L. C. & Brillantes, F. P. (1961) *Nature* **189,** 204-207.

21. Dao, T. L., Bock, F. G. & Greiner, M. J. (1960) *Journal of the National Cancer Institute* **25,** 991-1003.

22. Thordarson, G., Jin, E., Guzman, R. C., Swanson, S. M., Nandi, S. & Talamantes, F. (1995) *Carcinogenesis* **16,** 2847-53.

23. Sivaraman, L., Stephens, L. C., Markaverich, B. M., Clark, J. A., Krnacik, S., Conneely, O. M., O'Malley, B. W. & Medina, D. (1998) *Carcinogenesis* **19,** 1573-81.

24. Medina, D. & Smith, G. H. (1999) *Journal of the National Cancer Institute* **91,** 967-9.

25. Daniel, C. & Silberstein, G. (1987) in *The Mammary Gland: Development, Regulation, and Function.*, eds. Neville, M. & Daniel, C. (Plenum Press, New York), pp. 3-36.

26. Russo, J., Gusterson, B. A., Rogers, A. E., Russo, I. H., Wellings, S. R. & van, Z. M. (1990) *Laboratory Investigation* **62,** 244-78.

27. Russo, J., Rivera, R. & Russo, I. H. (1992) *Breast Cancer Research & Treatment* **23,** 211-8.

28. Russo, J. & Russo, I. H. (1993) *European Journal of Cancer Prevention* **2(Supplement 3),** 85-100.

29. Robinson, G. W., McKnight, R. A., Smith, G. H. & Hennighausen, L. (1995) *Development* **121,** 2079-2090.

30. Clarke, R., Dickson, R. B. & Lippman, M. E. (1992) *Critical Reviews in Oncology Hematology* **12,** 1-23.

31. Dickson, R. B. (1992) *Journal of Steroid Biochemistry & Molecular Biology* **41,** 389-400.

32. Silberstein, G. B., Flanders, K. C., Roberts, A. B. & Daniel, C. W. (1992) *Developmental Biology* **152,** 354-62.

33. Sternlicht, M. D., Lochter, A., Sympson, C. J., Huey, B., Rougier, J., Gray, J. W., Pinkel, D., Bissell, M. J. & Werb, Z. (1999) *Cell* **98,** 137-146.

34. Russo, J., Tay, L. K. & Russo, I. H. (1982) *Breast Cancer Research and Treatment* **2,** 5-73.

35. Gao, J., Ye, H. & Serrero, G. (2000) *J Cell Physiol* **182,** 297-302.

36. Kjeldsen, L., Johnsen, A. H., Sengelov, H. & Borregaard, N. (1993) *J Biol Chem* **268,** 10425-32.

37. Patarca, R., Saavedra, R. A. & Cantor, H. (1993) *Crit Rev Immunol* **13,** 225-46.

38. Schumann, R. R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S. & Ulevitch, R. J. (1990) *Science* **249,** 1429-31.

39. Denhardt, D. T. & Guo, X. (1993) *Faseb J* **7,** 1475-82.

40. Rajan, J. V., Marquis, S. T., Gardner, H. P. & Chodosh, L. A. (1997) *Developmental Biology* **184,** 385-401.

41. Marquis, S. T., Rajan, J. V., Wynshaw-Boris, A., Xu, J., Yin, G.-Y., Abel, K. J., Weber, B. L. & Chodosh, L. A. (1995) *Nature Genetics* **11,** 17-26.

42. Asai, K., Komine, Y., Kozutsumi, T., Yamaguchi, T., Komine, K. & Kumagai, K. (2000) *Vet Immunol Immunopathol* **73,** 233-40.

43. Tanneau, G. M., Hibrand-Saint Oyant, L., Chevaleyre, C. C. & Salmon, H. P. (1999) *J Histochem Cytochem* **47,** 1581-92.

44. Tatarczuch, L., Philip, C., Bischof, R. & Lee, C. S. (2000) *J Anat* **196,** 313-26.

45. Cornelius, L. A., Nehring, L. C., Harding, E., Bolanowski, M., Welgus, H. G., Kobayashi, D. K., Pierce, R. A. & Shapiro, S. D. (1998) *J Immunol* **161,** 6845-52.

46. Dong, Z., Kumar, R., Yang, X. & Fidler, I. J. (1997) *Cell* **88,** 801-10.

47. Dong, Z., Yoneda, J., Kumar, R. & Fidler, I. J. (1998) *J Exp Med* **188,** 755-63.

48. Belaaouaj, A., Shipley, J. M., Kobayashi, D. K., Zimonjic, D. B., Popescu, N., Silverman, G. A. & Shapiro, S. D. (1995) *J Biol Chem* **270,** 14568-75.

49. Strange, R., Li, F., Saurer, S., Burkhardt, A. & Friis, R. (1992) *Development* **115,** 49-58.

50. Tuck, A. B., Arsenault, D. M., O'Malley, F. P., Hota, C., Ling, M. C., Wilson, S. M. & Chambers, A. F. (1999) *Oncogene* **18,** 4237-46.

51. Ashkar, S., Weber, G. F., Panoutsakopoulou, V., Sanchirico, M. E., Jansson, M., Zawaideh, S., Rittling, S. R., Denhardt, D. T., Glimcher, M. J. & Cantor, H. (2000) *Science* **287,** 860-4.

52. Cooper, C. L. & Newburger, P. E. (1998) *J Cell Biochem* **71,** 277-85.

53. French, L. E., Soriano, J. V., Montesano, R. & Pepper, M. S. (1996) *Biol Reprod* **55,** 1213-20.

54. Jin, G. & Howe, P. H. (1999) *Eur J Biochem* **263,** 534-42.

55. Shi, X., Bai, S., Li, L. & Cao, X. (2001) *J Biol Chem* **276,** 850-5.

56. Fagenholz, P. J., Warren, S. M., Greenwald, J. A., Bouletreau, P. J., Spector, J. A., Crisera, F. E. & Longaker, M. T. (2001) *J Craniofac Surg* **12,** 183-90.

57. Nguyen, A. V. & Pollard, J. W. (2000) *Development* **127,** 3107-18.

58. Harris, J. R., Lippman, M. E., Veronesi, U. & Willett, W. (1992) *New England Journal of Medicine* **327,** 390-8.

59. Panico, L., D'Antonio, A., Salvatore, G., Mezza, E., Tortora, G., De Laurentiis, M., De Placido, S., Giordano, T., Merino, M., Salomon, D. S., Mullick, W. J., Pettinato, G., Schnitt, S. J., Bianco, A. R. & Ciardiello, F. (1996) *Int J Cancer* **65,** 51-6.

60. Normanno, N., Kim, N., Wen, D., Smith, K., Harris, A. L., Plowman, G., Colletta, G., Ciardiello, F. & Salomon, D. S. (1995) *Breast Cancer Res Treat* **35,** 293-7.

61. LeJeune, S., Leek, R., Horak, E., Plowman, G., Greenall, M. & Harris, A. L. (1993) *Cancer Res* **53,** 3597-602.

62. Niemeyer, C. C., Spencer-Dene, B., Wu, J. X. & Adamson, E. D. (1999) *Int J Cancer* **81,** 588-91.

63. Normanno, N., Selvam, M. P., Qi, C. F., Saeki, T., Johnson, G., Kim, N., Ciardiello, F., Shoyab, M., Plowman, G., Brandt, R. & et al. (1994) *Proc Natl Acad Sci U S A* **91,** 2790-4.

64. Ma, L., Gauville, C., Berthois, Y., Millot, G., Johnson, G. R. & Calvo, F. (1999) *Oncogene* **18,** 6513-20.

65. De Luca, A., Casamassimi, A., Selvam, M. P., Losito, S., Ciardiello, F., Agrawal, S., Salomon, D. S. & Normanno, N. (1999) *Int J Cancer* **80,** 589-94.

66. Luetteke, N. C., Qiu, T. H., Fenton, S. E., Troyer, K. L., Riedel, R. F., Chang, A. & Lee, D. C. (1999) *Development* **126,** 2739-50.

67. Fang, W., Hartmann, N., Chow, D. T., Riegel, A. T. & Wellstein, A. (1992) *J Biol Chem* **267,** 25889-97.

68. Kurtz, A., Spitzer, E., Zschiesche, W., Wellstein, A. & Grosse, R. (1998) *Biochem Soc Symp* **63,** 51-69.

69. Chauhan, A. K., Li, Y. S. & Deuel, T. F. (1993) *Proc Natl Acad Sci U S A* **90,** 679-82.

70. Zhang, N., Zhong, R., Wang, Z. Y. & Deuel, T. F. (1997) *J Biol Chem* **272,** 16733-6.

71. Souttou, B., Raulais, D. & Vigny, M. (2001) *J Cell Physiol* **187,** 59-64.

72. Choudhuri, R., Zhang, H. T., Donnini, S., Ziche, M. & Bicknell, R. (1997) *Cancer Res* **57,** 1814-9.

73. Papadimitriou, E., Polykratis, A., Courty, J., Koolwijk, P., Heroult, M. & Katsoris, P. (2001) *Biochem Biophys Res Commun* **282,** 306-13.

74. Hankinson, S. E., Willett, W. C., Colditz, G. A., Hunter, D. J., Michaud, D. S., Deroo, B., Rosner, B., Speizer, F. E. & Pollak, M. (1998) *Lancet* **351,** 1393-6.

75. Gorrin-Rivas, M. J., Arii, S., Furutani, M., Mizumoto, M., Mori, A., Hanaki, K., Maeda, M., Furuyama, H., Kondo, Y. & Imamura, M. (2000) *Clin Cancer Res* **6,** 1647-54.

76. Gorrin Rivas, M. J., Arii, S., Furutani, M., Harada, T., Mizumoto, M., Nishiyama, H., Fujita, J. & Imamura, M. (1998) *Hepatology* **28,** 986-93.

77. Zohar, R., Suzuki, N., Suzuki, K., Arora, P., Glogauer, M., McCulloch, C. A. & Sodek, J. (2000) *J Cell Physiol* **184,** 118-30.

78. Naot, D., Sionov, R. V. & Ish-Shalom, D. (1997) *Adv Cancer Res* **71,** 241-319.

79. Weber, G. F. & Cantor, H. (1996) *Cytokine Growth Factor Rev* **7,** 241-8.

80. Rajan, J. V., Wang, M., Marquis, S. T. & Chodosh, L. A. (1996) *Proceedings of the National Academy of Sciences, USA* **93,** 13078-13083.

81. Lockhart, D., Dong, H., Byrne, M., Follettie, M., Gallo, M., Chee, M., Mittmann, M., Wang, C., Kobayashi, M., Horton, H. & Brown, E. (1996) *Nature Biotechnology* **14,** 1675-1680.

FIGURE LEGENDS

Figure 1. Parity-induced morphological changes in mice and rats. Carmine-stained whole mounts (left 6 panels) from parous involuted from Sprague-Dawley rats and C57Bl/6 and FVB mice each display increased ductal branching as compared to the age-matched nulliparous gland. Magnification 25X. Histological analysis of hematoxylin and eosin-stained sections (right 2 panels) demonstrates that the proportion of epithelial cells is similar in the nulliparous and parous mammary glands. Magnification 400X.

Figure 2. Confirmation of parity-induced changes in gene expression. Northern hybridization analysis of gene expression for candidate genes identified by microarray analysis. Expression levels were determined for independent pools of mammary gland total RNA, each of which was derived from 15-20 age-matched nulliparous (lanes 1-3) or parous (lanes 4-6) animals. Genes downregulated as a consequence of parity included *superoxide dismutase III (SOD3), leptin (Ob), MUC18*, and *Islr*, a homologue of the Ig Superfamily. Genes upregulated by parity included *carbonic anhydrase isozyme II (CA2), adipocyte differentiation-related protein (ADFP), adenosine deaminase (ADA)*, and *carboxyl ester lipase (CEL).* Comparable epithelial cell content in nulliparous and parous mammary glands is demonstrated by equivalent levels of *CK18* expression when normalized to *β-actin* or 28S rRNA loading controls.

Figure 3. Differentially expressed genes identified by microarray analysis reproducibly distinguish between nulliparous and parous mammary tissues in mice and rats. (A) Six independent FVB samples (3 parous and 3 nulliparous pooled samples; 8-10 mice per pool) were

analyzed on Affymetrix MGU74A microarrays and clustered based on the expression patterns of genes identified as being differentially expressed in an independent set of parous and nulliparous FVB samples analyzed on Affymetrix Mu6500 microarrays. (B) Six independent Balb/c samples (3 parous and 3 nulliparous pooled samples; 3 mice per pool) were analyzed on Affymetrix MGU74A microarrays and clustered based on the expression patterns of genes identified as being differentially expressed in an independent set of parous and nulliparous FVB samples analyzed on Affymetrix Mu6500 microarrays. (C) Six Lewis rat samples (3 parous and 3 nulliparous pooled samples; 3-4 animals per pool) were analyzed on Affymetrix RGU34A microarrays. Genes identified as being differentially expressed in parous and nulliparous mice based on the analysis of Mu6500 microarray data were mapped via Homologene to the rat genome in order to perform clustering operations as above. (D) A subset of differentially expressed genes associated with the TGF-ß3 pathway were used to cluster the 12 murine nulliparous and parous samples described in A and B. (E) A subset of differentially expressed genes encoding epithelial growth factors were used to cluster the 6 rat samples described in C. Genes listed in Table 1 that were not represented on the MGU74A or RGU34A microarrays were omitted from the clustering analysis.

Figure 4. Differential expression of lymphoid and myeloid markers identify parity-induced changes in cell populations within the mammary gland. (A) Northern analysis of three independent pools of nulliparous (lanes 1-3) or parous (lanes 4-6) mammary gland total RNA isolated from FVB mice demonstrates parity-induced markers for macrophages (*MPEG1*), B-lymphocytes (*KLC*), and T-lymphocytes (*TDAG*). Hybridization to a probe for *β-actin* is shown as a loading control. (B) Northern hybridization of *MME, KLC* and *Eta-1* probes to total

mammary gland RNA isolated from female mice at the indicated developmental stages. Expression levels are compared to those of *β-actin* to account for dilutional effects due to large-scale increases in milk protein gene expression during late pregnancy and lactation. (C) *In situ* hybridization analysis of *MME, KLC* and *Eta-1* expression in the mammary gland at the indicated developmental stages. Bright-field (top) and dark-field (bottom) photomicrographs of murine mammary gland sections hybridized with ^{35}S-labeled *MME, KLC* and *Eta-1* specific antisense probes. No signal over background was detected in sections hybridized with sense probes (data not shown). Exposure times were identical for all dark-field photomicrographs of each gene to facilitate comparison of gene expression levels. Magnification 300x.

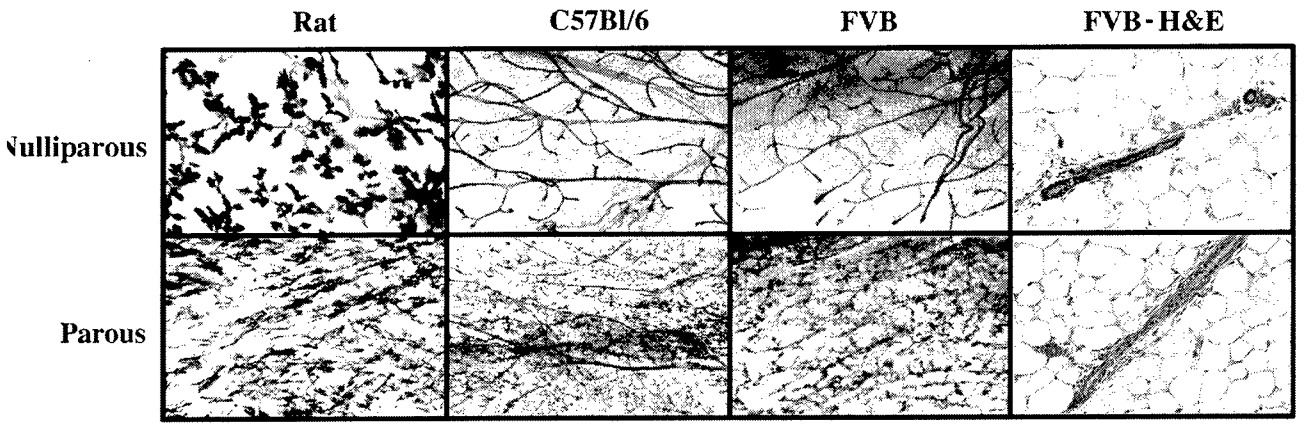
Figure 5. Parity-induced downregulation of amphiregulin and pleiotrophin expression in the mammary gland. (A) Northern hybridization analysis of three independent pools of nulliparous (lanes 1-3) or parous (lanes 4-6) mammary gland total RNA isolated from FVB mice demonstrates decreased expression of mitogenic signaling molecules. *β-actin* is shown as a loading control. (B) Northern hybridization analysis of the developmental patterns of *Areg* and *Ptn* expression in the mammary gland. Expression levels are compared to those of β-actin to account for dilutional effects due to large-scale increases in milk protein gene expression during late pregnancy and lactation. The 28S rRNA band is shown as a loading control. (C) *In situ* hybridization analysis of *Areg* expression at the indicated developmental stages. Bright-field (top) and dark-field (bottom) photomicrographs of murine mammary gland sections hybridized with an ^{35}S-labled *Areg*-specific antisense probe. No signal over background was detected in sections hybridized with a sense *Areg* probe (data not shown). Exposure times were identical for

all dark-field photomicrographs to facilitate comparison of gene expression changes. Magnification 300x.

Figure 6. Parity-induced increases in *TGF-β3* and *clusterin* expression in the mammary gland. (A) Northern analysis of three independent pools of nulliparous (lanes 1-3) and parous (lanes 4-6) mammary gland RNA isolated from FVB mice demonstrates increased expression of *TGF-β3* and *clusterin* in the parous gland. *β-actin* is shown as a loading control. (B) Northern hybridization analysis of the developmental patterns of *TGF-β3* and *clusterin* expression. Hybridization to *β-actin* is shown s control for loading and for dilutional effects of milk protein gene expression. (C) *In situ* hybridization analysis of *TGF-β3* and *clusterin* expression at the developmental stages. Bright-field (top) and dark-field (bottom) photomicrographs of murine mammary gland sections hybridized with ^{35}S-labeled *TGF-β3* or *clusterin* antisense probe. Magnification 300x.

Figure 7. Parity-induced changes in gene expression are permanent and conserved across species. A) Northern hybridization analysis for the indicated genes performed on pools of RNA from the mammary glands of parous animals that had undergone increasing periods of involution and of their age-matched nulliparous controls. Parous animals were mated at 4 wks of age, and underwent 21 days of lactation and either 4 wks, 16 wks, or 30 wks of postlactational involution. B) Probes for the indicated genes were hybridized to Northern membranes containing pools of total RNA from 3 parous (P) and 3 age-matched nulliparous (N) 129SvEv and Balb/c mice C) Probes for the indicated rat genes were hybridized to Northern membranes containing pools of total RNA from 10 parous (P) and 10 nulliparous (N) Sprague-Dawley rats. In each case, both

the direction and magnitude of change in expression between the nulliparous and parous states are comparable to that observed in FVB mice. β-actin and 28S rRNA are shown as a loading control.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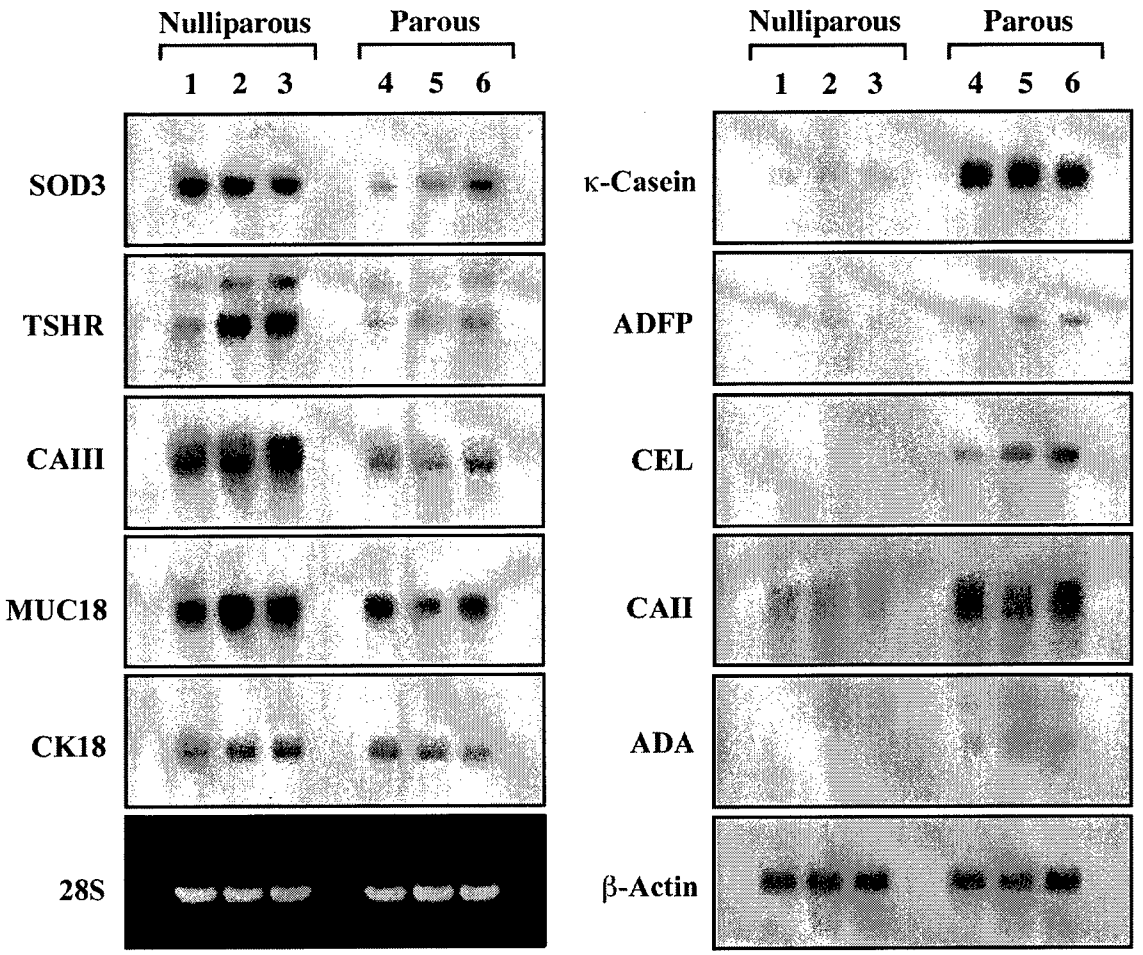
C57Bl/6
FVB
FVB - H&E
Nulliparous
Parous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SOD3
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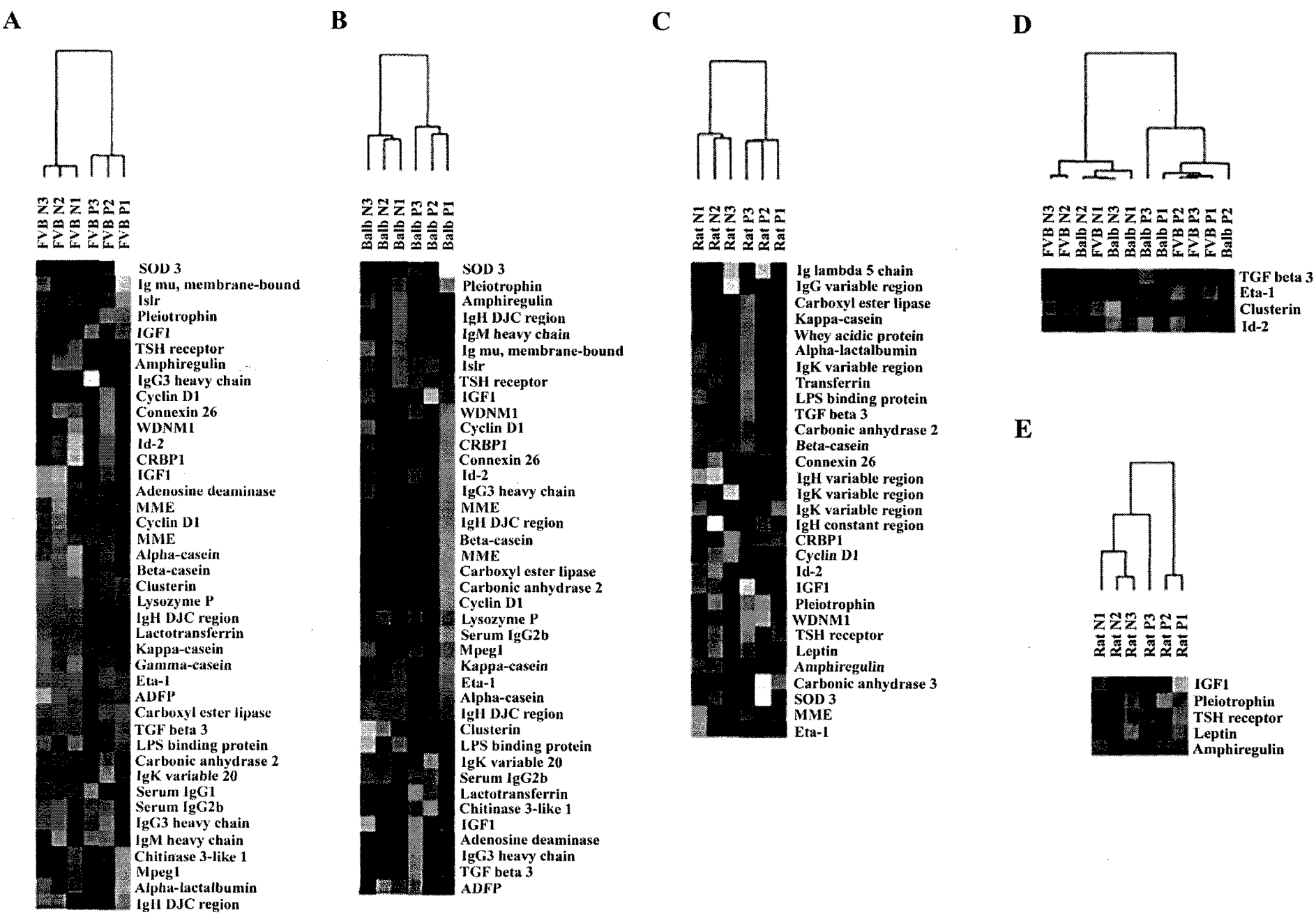
ADFP
CEL
CAII
ADA
β-Actin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FVB N2
FVB N1
FVB P3
FVB P2
FVB P1
SOD 3
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Islr
Pleiotrophin
IGF1
TSH receptor
Amphiregulin
IgG3 heavy chain
Cyclin D1
Connexin 26
WDNM1
Id-2
CRBP1
IGF1
Adenosine deaminase
MME
Cyclin D1
MME
Alpha-casein
Beta-casein
Clusterin
Lysozyme P
IgH DJC region
Lactotransferrin
Kappa-casein
Gamma-casein
Eta-1
ADFP
Carboxyl ester lipase
TGF beta 3
LPS binding protein
Carbonic anhydrase 2
IgK variable 20
Serum IgG1
Serum IgG2b
IgG3 heavy chain
IgM heavy chain
Chitinase 3-like 1
Mpeg1
Alpha-lactalbumin
IgH DJC region
B
Balb N3
Balb N2
Balb N1
Balb P3
Balb P2
Balb P1
SOD 3
Pleiotrophin
Amphiregulin
IgH DJC region
IgM heavy chain
Ig mu, membrane-bound
Islr
TSH receptor
IGF1
WDNM1
Cyclin D1
CRBP1
Connexin 26
Id-2
IgG3 heavy chain
MME
IgH DJC region
Beta-casein
MME
Carboxyl ester lipase
Carbonic anhydrase 2
Cyclin D1
Lysozyme P
Serum IgG2b
Mpeg1
Kappa-casein
Eta-1
Alpha-casein
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Clusterin
LPS binding protein
IgK variable 20
Serum IgG2b
Lactotransferrin
Chitinase 3-like 1
IGF1
Adenosine deaminase
IgG3 heavy chain
TGF beta 3
ADFP
C
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Rat N2
Rat N3
Rat P3
Rat P2
Rat P1
Ig lambda 5 chain
IgG variable region
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Kappa-casein
Whey acidic protein
Alpha-lactalbumin
IgK variable region
Transferrin
LPS binding protein
TGF beta 3
Carbonic anhydrase 2
Beta-casein
Connexin 26
IgH variable region
IgK variable region
IgK variable region
IgH constant region
CRBP1
Cyclin D1
Id-2
IGF1
Pleiotrophin
WDNM1
TSH receptor
Leptin
Amphiregulin
Carbonic anhydrase 3
SOD 3
MME
Eta-1
D
FVB N3
FVB N2
Balb N2
FVB N1
Balb N3
Balb N1
Balb P3
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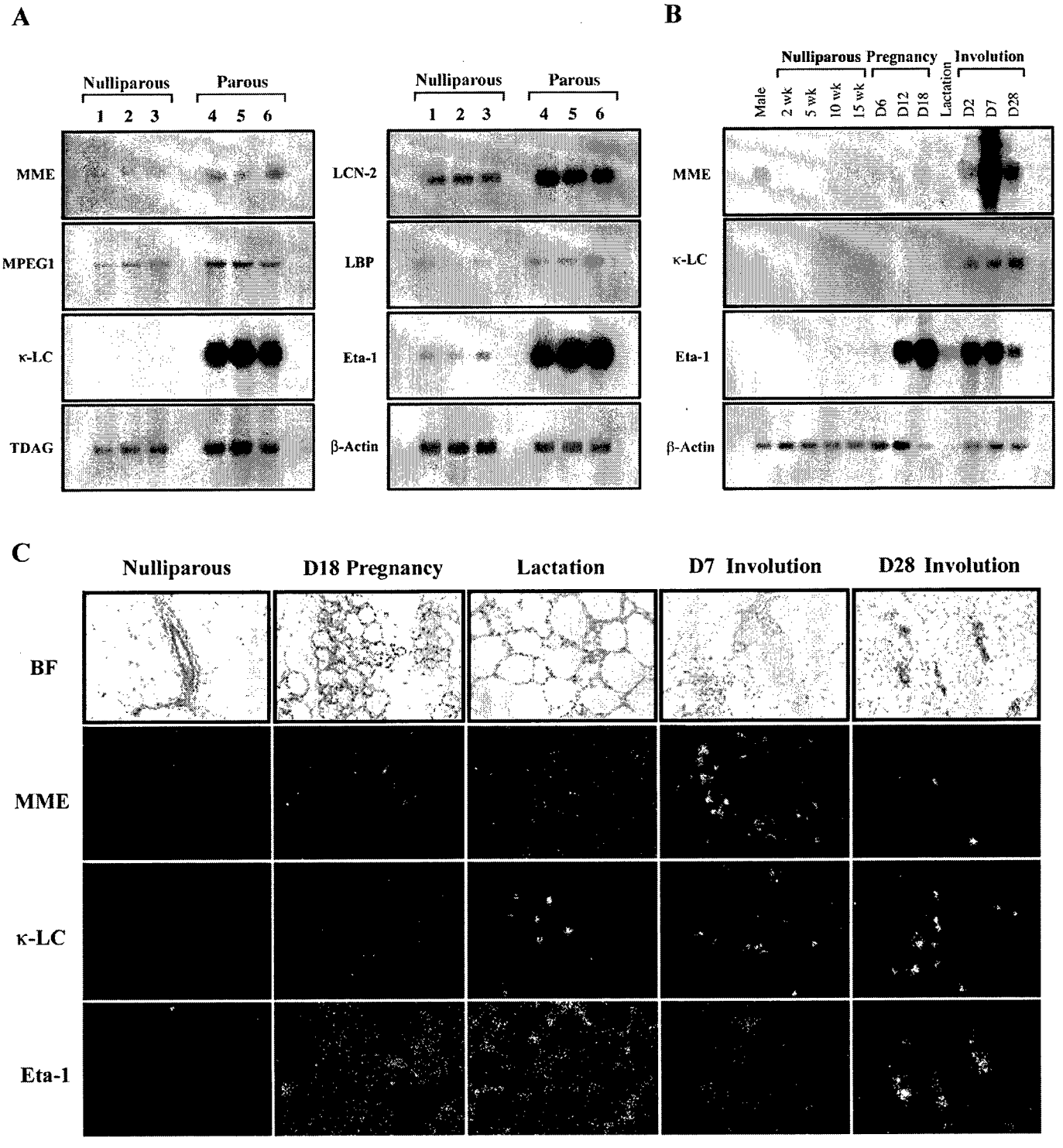
TSH receptor
Leptin
Amphiregulin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MPEG1
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TDAG
LCN-2
LBP
Eta-1
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B
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Pregnancy
Involution
Male
2 wk
5 wk
10 wk
15 wk
D6
D12
D18
Lactation
D2
D7
D28
MME
κ-LC
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Lactation
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D28 Involution
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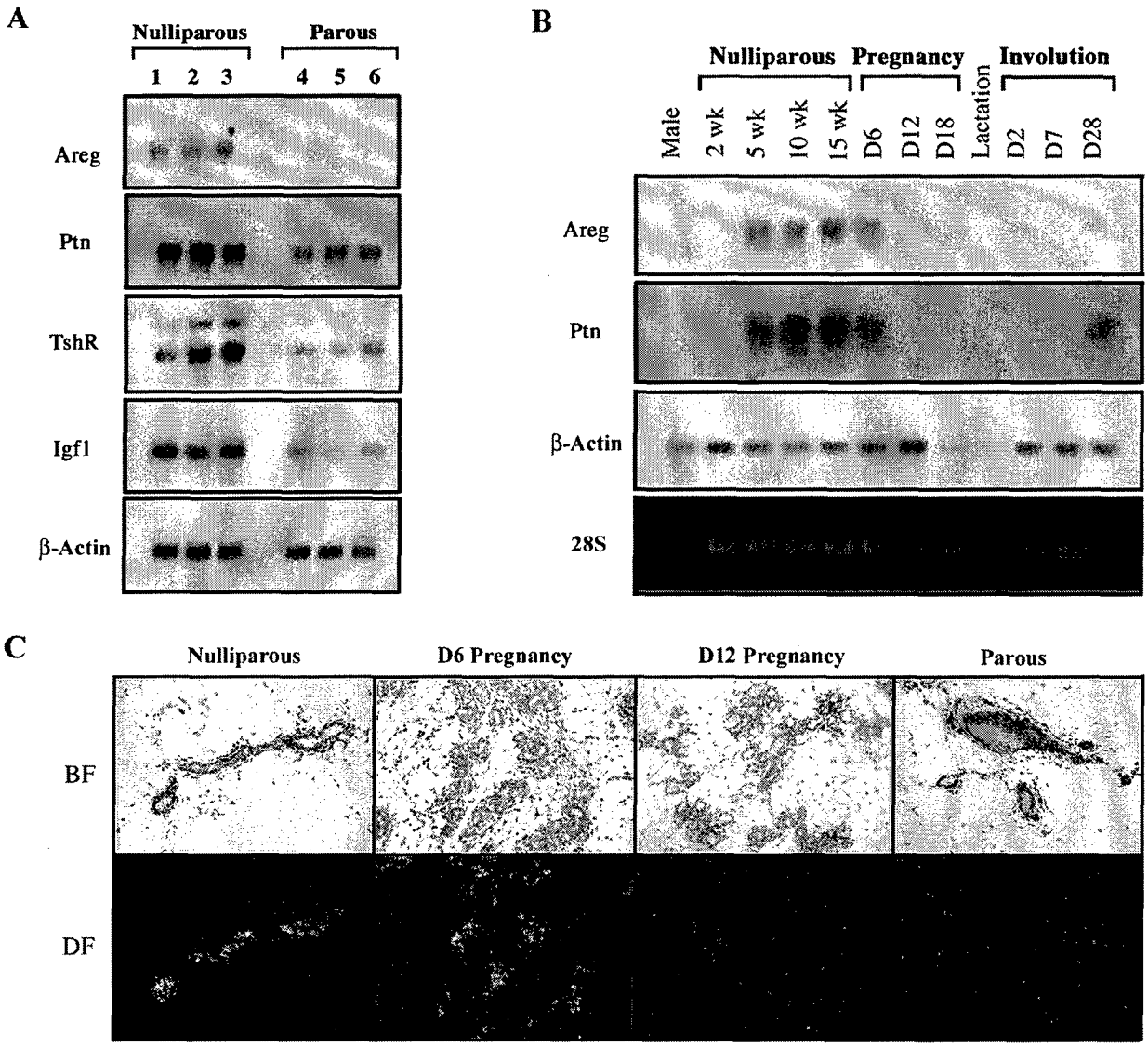
MME
κ-LC
Eta-1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1 2 3 4 5 6
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Ptn
TshR
Igf1
β-Actin
B
Nulliparous
Pregnancy
Involution
Male
2 wk
5 wk
10 wk
15 wk
D6
D12
D18
Lactation
D2
D7
D28
Areg
Ptn
β-Actin
28S
C
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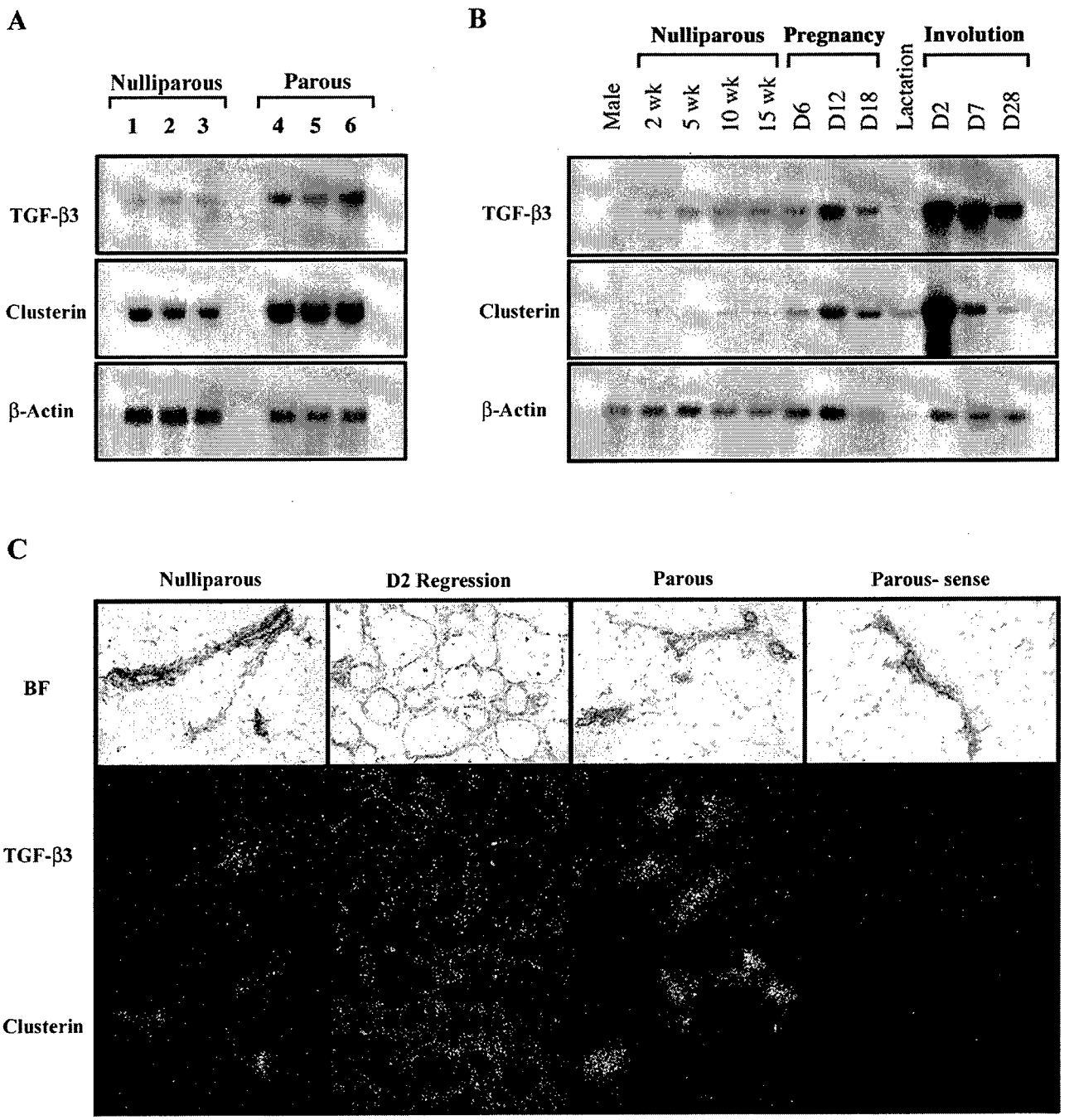
D6 Pregnancy
D12 Pregnancy
Parous
BF
DF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Parous
1 2 3
4 5 6
TGF-β3
Clusterin
β-Actin
B
Nulliparous
Pregnancy
Involution
Male
2 wk
5 wk
10 wk
15 wk
D6
D12
D18
Lactation
D2
D7
D28
TGF-β3
Clusterin
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C
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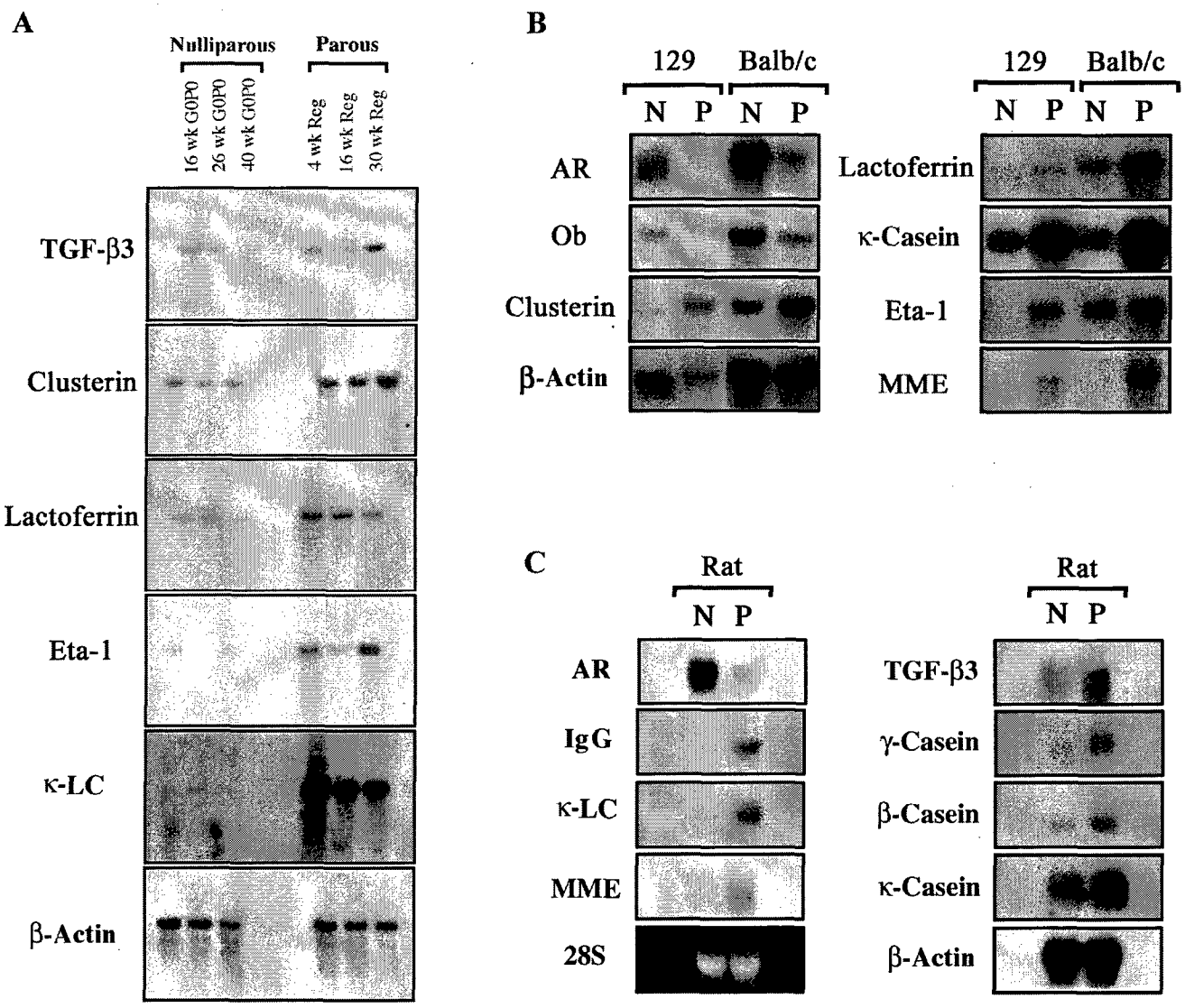
D2 Regression
Parous
Parous- sense
BF
TGF-β3
Clusterin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Parous
16 wk G0P0
26 wk G0P0
40 wk G0P0
4 wk Reg
16 wk Reg
30 wk Reg
TGF-β3
Clusterin
Lactoferrin
Eta-1
κ-LC
β-Actin
B
129
Balb/c
N P N P
AR
Ob
Clusterin
β-Actin
129
Balb/c
N P N P
Lactoferrin
κ-Casein
Eta-1
MME
C
Rat
N P
AR
IgG
κ-LC
MME
28S
Rat
N P
TGF-β3
γ-Casein
β-Casein
κ-Casein
β-Actin

Table 1: Differentially Expressed Genes

Genes Down-Regulated

Sequence Identity	Accession Number	Function	Avg. Fold Change	Northern
Amphiregulin	L41352	Growth Factor	2.8	Y
Pleiotrophin	D90225	Growth Factor	2.0	Y
Insulin-Like Growth Factor 1B	W10072	Growth Factor	2.2	ND
Insulin-Like Growth Factor 1A	X04480	Growth Factor	1.6	Y
Thyroid Stimulating Hormone Receptor	U02602	Hormone Receptor	2.0	Y
Leptin	U18812	Hormone	2.0	Y
Ig Superfamily Containing Leucine-rich repeat	AA059664	Cell Adhesion	2.5	Y
MUC 18	AA088962	Cell Adhesion	2.0	Y
Superoxide Dismutase III (SOD3)	X84940	Oxidoreductase	2.5	Y
Carbonic Anhydrase III	M27796	Hydratase	1.4	Y

Genes Up-Regulated

Sequence Identity	Accession Number	Function	Avg. Fold Change	Northern
Whey Acidic Protein	J00544	Differentiation/Milk Protein	24	Y
Gamma-Casein	D10215	Differentiation/Milk Protein	20	Y
Alpha-Lactalbumin	M80909	Differentiation/Milk Protein	13	Y
Lactoferrin	J03298	Differentiation/Iron Transport	4.8	Y
Alpha-Casein	M36780	Differentiation/Milk Protein	4.2	ND
WDNM1	X93037	Differentiation/Protease Inhibitor	3.5	Y
Beta-Casein	X04490	Differentiation/Milk Protein	3.3	Y
Kappa-Casein	M10114	Differentiation/Milk Protein	2.9	Y
Adipocyte Differentiation Related Protein	M93275	Differentiation	1.8	Y
Carboxyl Ester Lipase	U37386	Lipid Degradation	6.6	Y
LPS-Binding Protein	X99347	Antibacterial/Milk	6.5	Y
Lysozyme P	M21050	Antibacterial/Milk	2.0	ND
Immunoglobulin M Heavy Chain	ET61785	Immunoglobulin	30	Y
Immunoglobulin G Heavy Chain	ET61798	Immunoglobulin	13	Y
Immunoglobulin A Heavy Chain	J00475	Immunoglobulin	21	Y
Kappa Light Chain	X16678	Immunoglobulin	7.0	Y
Macrophage Metalloelastase	M82831	Metalloprotease	2.1	Y
Macrophage Expressed gene 1 (Mpeg1)	L20315	Cell Signalling	1.7	Y
Lipocalin 2	W13166	Cell Signalling	2.6	Y
T-cell death associated gene (Tdag)	U44088	Cell Signalling	2.3	Y
Transforming Growth Factor Beta-3	M32745	Growth Inhibition	1.9	Y
Clusterin	L08325	Apoptosis/TGF-ß Pathway	2.0	Y
Eta-1	X16151	Cell Signalling/TGF-ß Pathway	8.8	Y
Id-2	M69293	Cell Cycle/TGF-ß Pathway	2.3	Y
Carbonic Anhydrase II	K00811	Hydratase	3.0	Y
Cyclin D1	A1849928	Cell Cycle	1.8	Y
CRBP1	X60367	Retinol-Binding	3.5	Y
Connexin 26	M81445	Gap Junction	3.1	Y
Folate-Binding Protein 1	ET63126	Receptor	2.6	Y
Adenosine Deaminase	M10319	Nucleotide Metabolism	3.0	Y
Chitinase 3-like 1 (BRP-39)	X93035	Glycoprotein	3.2	Y

Table 1: Genes found to exhibit a persistent change in expression in fully involuted compared to age-matched nulliparous mammary glands. Genes whose differential expression was confirmed by Northern analysis are indicated (Y). Genes whose differential expression was not tested (ND) by Northern hybridization were listed if they displayed a consistent change in regulation in 3/3 microarray experiments.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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Exel